The influence of IL-10 on dendritic cell activation

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

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Special thanks to my family, especially our Adam, for all their love and laughter.

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For my dad,

who died too soon.

Ode to dendritic cells

'What is a dendritic cell, Daddy?'
'A dendritic cell, Son,' said I,
'Is a tall bag of cheese
Plus a Chinaman's knees
And the leg of a nanny goat's eye.'

'How strange is a dendritic cell, Daddy?'
'As strange as strange,' I replied.
'When the sun's in the West
It appears in a vest
Sailing out with the noonday tide.'

'What shape is a dendritic cell, Daddy?'
'The shape, my son, I'll explain:
It's tall round the nose
Which continually grows
In the general direction of Spain.'

'Are you sure there's a dendritic cell, Daddy?'
'Am I sure, my son?' said I.
'Why, I've seen it, not quite
On a dark sunny night
Do you think that I'd tell you a lie?'

With apologies to Spike Milligan and his *Bongaloo*

Abstract

Dendritic cells (DCs) are the watchmen of the immune system. They survey the peripheral tissues and display samples of surrounding antigens for inspection by T cells. Pathogenic or inflammatory signals trigger DCs to mature, upregulating their expression of MHC and costimulatory molecules and converting them into potent T cell stimulators. The character of the activated DC depends not only on the stimulus but also on concomitant environmental signals. This thesis tests the hypothesis that *in vitro* manipulation of DC function can be used to direct immune responses *in vivo*. The work focuses on the cytokine IL-10 and asks whether its impact on DC maturation can mediate T cell tolerance.

IL-10 has been reported to trap DCs in an immature state, leading to antigen presentation without full costimulation and consequent T cell anergy. Data presented here show that DCs do become activated in the presence of IL-10. They downregulate antigen uptake and, 6 hours after stimulation, display high levels of MHCII and B7. Their activation is short-lived, with both MHC and B7 expression returning to baseline within 18h. Even at 6 hours, with high levels of surface MHC and costimulation, the IL-10 treated DCs express little IL-12 and fail to elicit strong T cell proliferation. IL-10 seems not to act by inhibiting DC maturation but instead by dictating the kinetics and quality of their activation.

The consequence of this DC activation for the responding T cells is also examined. Both *in vitro* and *in vivo*, using co-cultures and adoptive transfers of TCR transgenic T cells followed by DC-based immunisation, initial contact with IL-10 treated DCs appears to leave T cells hyporesponsive to subsequent challenge. In a mouse model of autoimmunity, these DCs suppress disease.

Taken together these data suggest that, rather than preventing DC maturation, IL-10 directs an active DC phenotype that can regulate immune responses. These DCs have exciting therapeutic potential.

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Abbreviations

APC	antigen presenting cell
B7.1, B7.2	CD80 and CD86, respectively
BBB	blood brain barrier
BMDC	bone marrow – derived dendritic cell
BOB	blood ocular barrier
CFA	complete Freund's adjuvant
CNS	central nervous system
DC	dendritic cell
DO11.10	a transgenic TCR specific for the 323-339 peptide epitope of ovalbumin
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme linked immunosorbent assay
GM-CSF	granulocyte / macrophage colony stimulating factor
H&E	haematoxylin and eosin
i.p.	intraperitoneal
i.v.	intravenous
IBD	inflammatory bowel disease
ICOS	inducible costimulator
ITAM	immunoreceptor tyrosine-based activation motif
IRAK	IL-1 receptor – associated kinase
KJ126	a monoclonal antibody specific for the DO11.10 TCR
LC	Langerhans cell
LPS	lipopolysaccharide
MACS	magnetically activated cell sorting
MAP kinase	mitogen-activated protein kinase
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NOD	non obese diabetic
PAMPs	pathogen associated molecular patterns
PGE_2	prostaglandin E2
PLP	proteolipid protein
PRRs	pattern recognition receptors
RT-PCR	reverse transcriptase – polymerase chain reaction
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SMAC	supramolecular activation cluster, the DC:T cell synapse
TCR	T cell receptor
TLR	Toll-like receptor
TRAF	TNF receptor associated factor

Chapter 1 - Introduction

1.1 Overview

Dendritic cells (DCs) are potent antigen presenting cells (APCs). *In vitro* they stimulate T cell proliferation with 100-1000 times the efficiency of bulk leukocytes [1]; *in vivo* they are uniquely capable of eliciting a primary antigen specific response [2, 3]. DCs are often described as the sentry guards of the immune system, stationed throughout the periphery, continuously sampling their environment by phagocytosis and pinocytosis [4, 5]. Antigens are taken in, processed and presented on the cell surface. As the DCs migrate to the draining lymph node, they upregulate MHC and costimulatory molecules and mature into specialised T cell stimulators, initiating adaptive immunity [6, 7].

Their central role in the activation of naïve T cells gives DCs a strategic position in the control of immune responses. Many DC phenotypes exist, reflecting differences in ontogeny, maturation stage and environment. Both myeloid and lymphoid DCs have been reported and multiple developmental pathways are described (reviewed in [8, 9]). Evidence suggests that DC phenotype can be altered by certain cytokines and a model has been proposed in which different immune responses are thought to result from equivalent DCs matured in different conditions [10]. DCs are capable both of influencing the Th1/Th2 balance [11, 12] and inducing either tolerance or immunity [13, 14]. An appreciation of the mechanisms involved in this control might enable specific manipulation of the immune system. This project set out to examine the flexibility of DC function, using DCs cultured in the presence of defined stimuli. The hypothesis is that DCs modified by stimulation *in vitro* can be used to direct the outcome of immune responses *in vivo*.

1.2 Professional APCs

DCs were first observed by Langerhans in 1868 [15], but it was not until 1973 that Steinman and Cohn provided a full description [16]. They were identified initially by morphology (fig 1.1): their long cytoplasmic pseudopodia distinguished them from other large mononuclear cells within a spleen preparation. Electron microscopy revealed a typically large nucleus, an electron lucent cytoplasm relatively devoid of small particles and a smooth outer surface, quite different from the ruffled appearance of the macrophage membrane. Vital dyes and histochemical reagents also demonstrated characteristic staining patterns. Dendritic cells were declared a novel cell type, related to but distinct from macrophages [16-19].

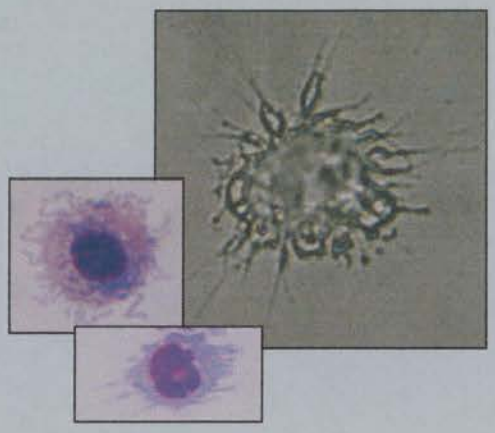

Figure 1.1 DC morphology. The main picture shows a DC grown in a glass chamber well and photographed using phase contrast microscopy, and was provided by Lynda Stuart (CIR, University of Edinburgh). The smaller pictures are cytospins, stained with H&E. These DCs were grown from bone marrow precursors (upper picture; see section 2.3.3) or isolated from spleen (lower picture; section 2.3.2) and were photographed at magnification x1000.

1.2.1 Antigen uptake

Subsequent use of immunocytochemistry and flow cytometry revealed variations in DC maturation state [20, 21]. Immature cells such as Langerhans cells (LCs) in the skin and interstitial DCs in the kidneys, heart and other peripheral tissues [22, 23] are primed for antigen acquisition. They filter soluble antigens from extracellular fluid taken up by macropinocytosis at such a rate that they can internalise the equivalent of their own volume within two hours [4]. They are highly phagocytic, engulfing particulate antigens or whole bacteria via membrane-bound receptors [24]. Phagocytosis is enhanced by opsonisation of pathogens with antibody or complement [25, 26] and immature DCs have been shown to express both Fc receptors (FcγRI, RII, and RIII and FcεRI and RII) [27] and the receptor for C3b [28]. The CD11c marker often used to identify DCs is a component of CR4 [29]. Scavenger receptors such as CD36 are also expressed by immature DCs, enabling the phagocytosis of apoptotic cells or debris [30, 31].

Immature DCs also capture considerable amounts of antigen by receptor-mediated endocytosis [32]. They possess several lectin receptors, including the DC-specific ICAM-grabbing non-integrin (DC-SIGN) [33], the DC immunoreceptor (DCIR) [34] and DEC-205 [35, 36]. The natural ligands for DEC-205 are unknown, but anti-DEC antibodies are internalised and presented to T cells with 100 times the efficiency of non-specific antibodies [37]. DCs also express the macrophage mannose receptor, which binds carbohydrates and targets them to lysosomes [38]. The low pH of the lysosomal compartment dissociates the complexes and the receptor then returns to the cell surface. This rapid recycling allows a limited number of molecules to internalise a considerable amount of antigen [4]. Interestingly, LCs express much lower levels of the mannose receptor than other immature DCs and their endocytic and phagocytic activity is relatively impaired [39]. This may be a regulatory mechanism to dampen reactivity to harmless environmental antigens that contact the skin.

The binding and internalisation of antigens is further enhanced by DC receptors that recognise common molecular structures on the surface of invading microorganisms.

Janeway first proposed the existence of pathogen-associated molecular patterns (PAMPs) and complementary pattern recognition receptors (PRRs) before any examples were identified; he argued that such a system was necessary to distinguish self from non-self and to prevent autoreactivity of the immune system [40]. Since immunity encourages antigenic variation by selecting against pathogens bearing recognised motifs, PAMPs are usually structures that are essential for microbial survival and hence are shared by many different microorganisms. They include cell wall components of yeasts, gram negative and gram positive bacteria, such as mannans, lipopolysaccharides (LPS), lipoteichoic acids (LTA) and peptidoglycans (PDG), as well as flagellin, bacterial CpG DNA motifs and the double-stranded RNA of viruses [41]. Their receptors encompass antigen binding molecules such as DEC-205 and the mannose receptor, together with a recently discovered family of specialised PRRs, the Toll-like receptors (TLRs).

The original Toll receptor is a Drosophila protein involved in body patterning and anti-fungal immunity [42]. Ten mammalian homologues have now been identified, expressed on APCs and other cell types [43, 44] and able to recognise a wide range of microbial products (table 1.2). The TLRs are implicated in antigen internalisation: CpG triggering of TLR9 requires an endosomal location [45, 46] and TLR2 is recruited to phagosomes after challenge with zymosan [47]. Both TLR2 and TLR6 accumulated in endosomes containing phagocytosed red blood cells, however, despite neither being a specific receptor [48]. This may reflect a passive role of the TLRs as innocent bystanders, swept into the phagosomes by physical proximity to the receptor actually engaged by the erythrocytes. Alternatively, TLRs may be actively recruited to the phagosome after internalisation, enabling them to peruse the phagosomal contents and transmit danger signals when appropriate [48]. This ability to signal is the basis of much of the interest currently surrounding TLRs: not only do they bind pathogens but, by concomitantly stimulating DCs, they also form a bridge between the innate and adaptive immune systems [44].

TLR	Putative ligand	Reference
1	unknown, but dimerises with TLR2	[49]
2	lipoproteins, peptidoglycans, structural variants of LPS, zymosans	[48, 50-53]
3	double stranded RNA	[54]
4	LPS, F protein of RSV, HSP-60, fibronectin	[55, 56] [57] [58, 59]
5	flagellin	[60]
6	unknown, but dimerises with TLR2	[48, 49]
7	unknown	[61]
8	unknown	[61]
9	CpG DNA	[46]
10	unknown	[62]

Table 1.2 Mammalian Toll-like receptors and their ligands. Compiled from [44, 63].

1.2.2 DC stimulation

The acquisition of foreign antigens is closely associated with DC activation, initiating the changes that convert the DC from a dedicated antigen collector into a specialised presenting cell. As pathogens are bound and internalised, they stimulate signalling cascades that conventionally lead to upregulation of MHC and costimulatory molecules and the release of proinflammatory cytokines [7]. The TLRs are classic examples of these signalling molecules. The inflammatory response to most LPS molecules is mediated via TLR4: LPS hyporesponsive C3H/HeJ mice carry a mutated version of the *tlr4* gene [55] and B cells from $TLR4^{-/-}$ mice fail to proliferate or to upregulate MHCII after LPS stimulation, despite a

normal reaction to IL-4 [56]. TLR4 deficient macrophages are similarly affected, releasing neither TNFα nor nitric oxide in response to LPS stimulation [56].

The TLR4 receptor is a transmembrane homodimer which clusters with both CD14 and an adaptor molecule, MD-2, to form an LPS activation complex on the DC surface (fig 1.3)[64, 65]. Signals are transmitted into the cell through one of two adaptor molecules that bind to a Toll/IL-1R (TIR) domain in the cytoplasmic tail of each TLR4 molecule [66, 67]. MyD88 acts in conjunction with another adaptor, Tollip, to recruit the IL-1 receptor associated kinase (IRAK) [68]. Once activated, IRAK dissociates from the complex and signals via a TNF-receptor associated factor, TRAF 6, to initiate a MAP Kinase cascade that results in dephosphorylation of IκB, release of the NFκB transcription factor and expression of a variety of genes under

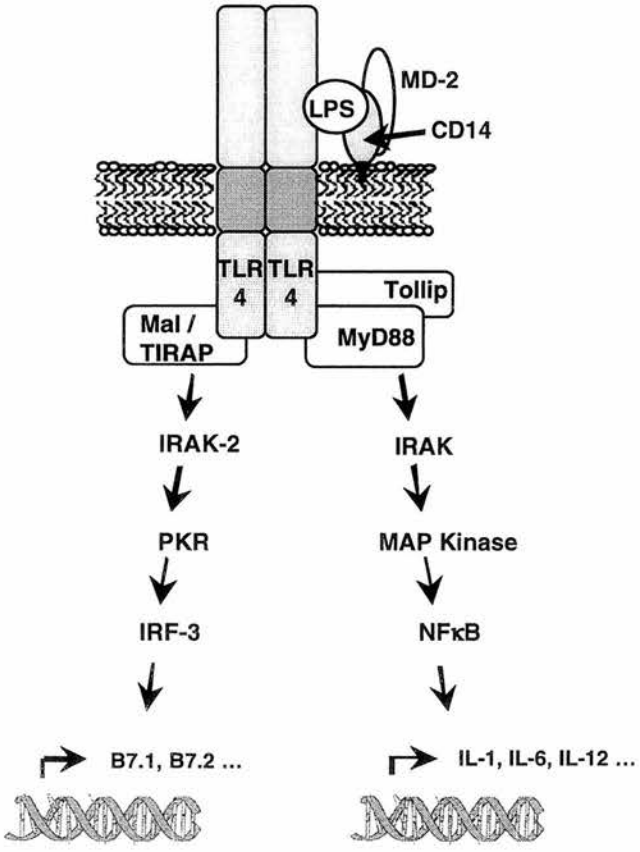

Figure 1.3 Signalling through TLR4. LPS binds the TLR4 homodimer through interaction with the coreceptor CD14 and an adaptor protein, MD-2. Intracellular signalling then occurs either via the adaptor molecules MyD88 and Tollip, which activate the IL-1 receptor associated kinase (IRAK) and initiate a mitogen activated protein (MAP) Kinase cascade to release NFκB from suppression, or via the alternative adaptor MyD88-adaptor like (Mal, also known as TIRAP) which then signals through IRAK-2 and protein Kinase R (PKR) to activate the transcription factor IRF-3.

the control of NFκB response elements, including those encoding IL-1, IL-6 and IL-12 [69, 70]. The second adaptor, named both MyD88 adaptor-like (Mal) and TIR domain-containing adaptor protein (TIRAP) [71, 72], provides a MyD88 independent pathway that leads to activation of the transcription factor IFN-regulated factor 3 (IRF-3) and expression of genes such as B7.1 and B7.2 [73, 74].

MyD88 and Tollip also associate with other TLR receptors, including TLR2, 5, 9 and their heterodimers [48], and several of the downstream molecules such as IRAK and the TRAF family serve additional receptors on the DC surface [75-77]. This convergence of signalling pathways at least partly explains the similarity of DC activation in response to quite different stimuli. Huang *et al.* used microarray technology to demonstrate that exposure of DCs to *Escherichia coli*, *Candida albicans* and influenza virus each induced the same set of 166 genes, and that this effect could be reproduced almost exactly by replacing the *E.coli* with LPS and the influenza virus with double stranded RNA [78]. This suggests that TLR4 and TLR3 use a common signalling pathway, presumably targeting NFκB [78]. In addition to the shared 166 genes, however, *E.coli* induced another 118 and influenza virus activated a specific 58. These differences reflect the ability of alternative adaptor molecules and coreceptors to interact with the TLRs, translating distinct stimuli into individual blends of genes and appropriately tailored DC phenotypes [79].

The ability of TLRs to bind microbial motifs is a prime example of self:non-self discrimination, but their specificity is not absolute. Both human heat shock protein 60 and fibronectin have been shown to activate signalling through endogenous TLR4 [58, 59]. Interestingly, fibronectin interacts with TLR4 via its extra domain A (EDA) region. The EDA region is normally absent and is only expressed as a product of alternative splicing, induced by tissue injury [80, 81]. The TLRs appear then to be capable of recognising both foreign invasion and signs of damage or 'danger' within the host [82].

DC activation can also be triggered by other endogenous molecules. Necrotic cells are potent stimuli of DCs, reflecting both their active production of distress signals such as heat shock proteins and the passive release of molecules previously

sequestered within the cell [83]. In the skin, IL-1, GM-CSF and TNFα are all secreted by keratinocytes in response to physical or chemical disruption of the epidermis, and all induce activation of local LCs [84-86]. Indeed, Bennett *et al.* proposed that the DC activation and subsequent immune reaction caused by infection with *Leishmania mexicana* is initiated not by the uptake of parasites but rather by IL-1 or other endogenous stimuli released in response to the bite of the sandfly vector [87].

1.2.3 DC migration

The Langerhans Cell is often quoted as an illustration of DC biology [88]. DC precursors enter the skin from the blood, seeding the tissue with a network of immature DCs [89]. Antigen or inflammation elicits a rapid, local recruitment of these immature DCs [90], and as the cells respond to the activation signals that they encounter, they undergo a series of changes in phenotype and function known as maturation [91]. They show a transient upregulation of antigen acquisition [92], ensuring that the antigens they carry reflect the microenvironment at the point of DC activation, before migrating out of the skin, via the lymphatics to the T cell area of the draining lymph node. As they travel, DCs upregulate expression of MHC and costimulatory molecules, and they arrive as powerful T cell stimulators [7].

The emigration of LCs from the skin and their entry to the lymph nodes is controlled by adhesion molecules and chemokine gradients. Immature LCs are anchored to surrounding keratinocytes by their expression of E-cadherin [93]. On activation, E-cadherin is rapidly downregulated [94] and the LCs instead display receptors for α_6/β_1 and α_6/β_4 integrins [95], encouraging adhesion to the basement membrane. LCs are capable of protolytic activity and secrete type IV collagenase [96]. This enables them to penetrate the basement membrane and travel through the dermis to the lymphatic system. The shifting attachments between LCs and connective tissue are also mediated by changes in the CD44 isoforms expressed by the LCs, a

consequence of alternative splice variations used at different stages of maturation [25, 97].

The role of chemokines in directing the movement of DCs is also becoming increasingly well understood [98]. Chemokines are small, chemoattractive proteins, often named after the number and spacing of cysteine (C) residues at their N-terminus [99]. Immature LCs are recruited to sites of inflammation by their expression of the chemokine receptors CCR1, 2, 5 and 6, which recognise molecules such as macrophage inflammatory protein (MIP)-1α, MIP-1β and regulated on activation, normal T cell expressed and secreted (RANTES). LC activation initiates the downregulation of these receptors and replaces them with high levels of CCR7 which, through its attraction to the chemokines secondary lymphoid-tissue chemokine (SLC) and MIP-3β, guides the LCs into the T cell areas of draining lymph nodes [100, 101]. The importance of this interaction is illustrated in mice lacking either CCR7 or SLC: both knockout strains are unable to mount primary immune responses because of inefficient recruitment of DCs and naïve T cells into secondary lymphoid tissue [102, 103].

Once in the lymph node, the potent ability of DCs to stimulate T cells is also a result of their maturation. The transient increase in antigen acquisition following LC stimulation is accompanied by a temporary accumulation of *de novo* synthesised MHCII molecules in cytoplasmic vesicles, enabling efficient loading with external antigens [4, 92]. This ends with a marked downregulation of both antigen uptake and MHC production and instead, existing MHC:peptide complexes are transported to and stabilised on the cell surface [92, 104]. This enhanced antigen presentation is supplemented by increased expression of adhesion molecules including ICAM-1, 2 and 3 and DC-SIGN, which together strengthen the physical interaction between DCs and T cells [33, 105], and by marked upregulation of costimulatory molecules such as B7.1 (CD80), B7.2 (CD86) and CD40, which augment T cell stimulation [106]. DC maturation also elicits increased secretion of cytokines that promote effector T cell development, including IL-12, IFNγ and perhaps even IL-2 [107, 108] [109]. As a result, the classical outcome of LC activation and maturation is T cell immunity (fig 1.4)[110].

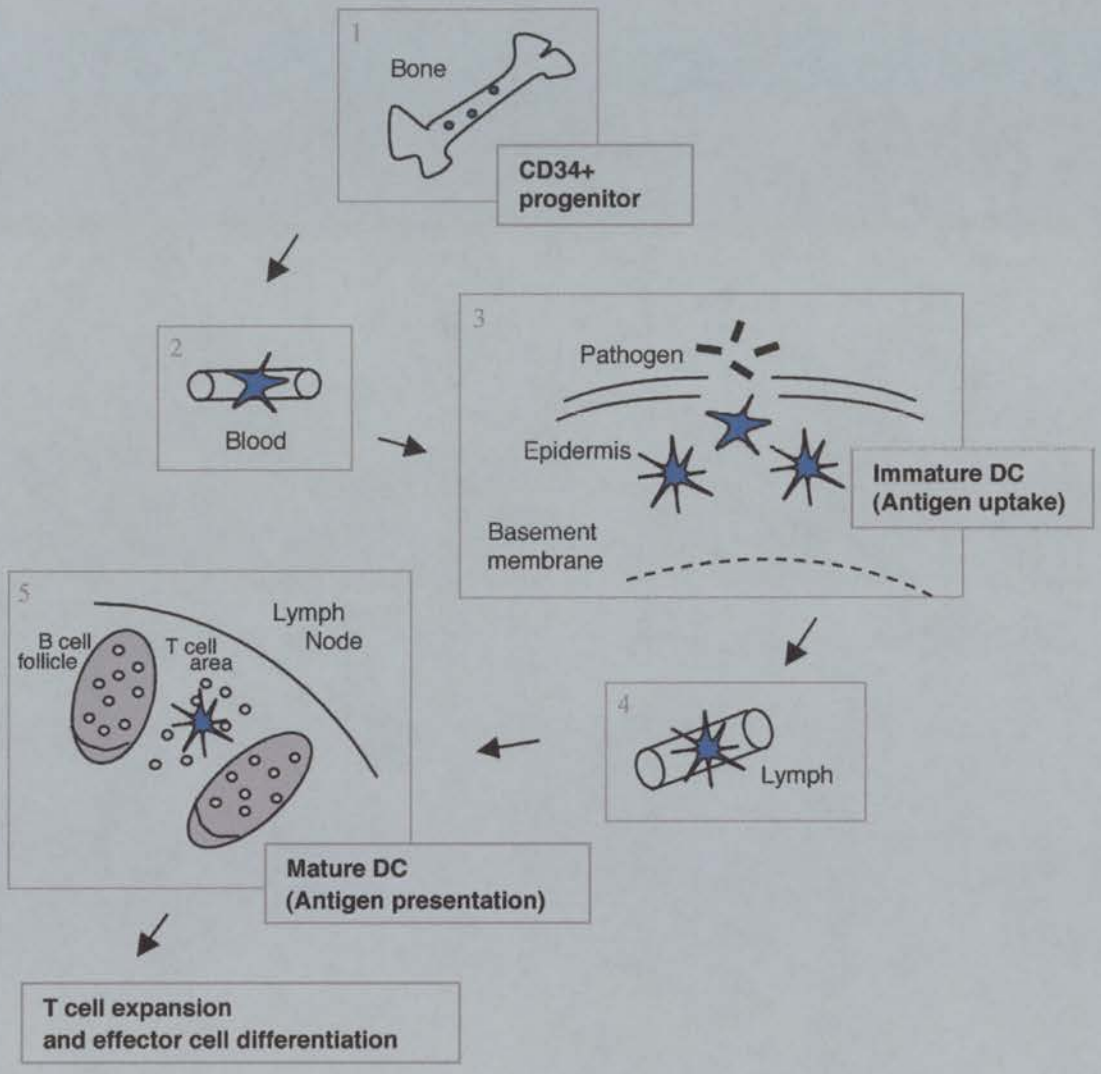

Figure 1.4 Life cycle of a Langerhans Cell. Haematopoietic progenitors seed the tissues with immature DCs. Inflammation or antigen stimulates the DC, triggering maturation and migration to the draining lymph node. There the mature DC stimulates a vigorous T cell response. Adapted from [398].

1.2.5 Activation vs maturation

Models of DC maturation such as this provide considerable insight into the important stages in DC development, but they also raise several questions. Do all DCs respond to stimulation in this way? Is maturation an inevitable consequence of DC activation? The terminology here is confusing: 'activation' and 'maturation' are often used interchangeably and neither has a clear definition. Strong stimuli such as LPS or heat-killed bacteria, commonly used to activate DC *in vitro*, cause DC maturation and subsequent death [5]. In this situation, the two words describe very similar events. This is not always the case. Both recombinant TNFα and a *Bordatella pertussis* antigen have been reported to upregulate MHCII and B7 expression on DCs without eliciting cytokine secretion [111, 112]. The DCs are clearly activated, and yet they have only completed part of the classical maturation process [113]. A more extreme example is provided by DCs cultured overnight in soluble egg antigen (SEA), derived from the eggs of the parasitic helminth *Schistosoma mansoni*. Mice infected with the intact parasite mount a strong Th2 response that is essential for survival [114]. DCs treated with SEA and then injected *in vivo* induce a similarly powerful Th2 reaction. Importantly, they achieve this without any detectable increase in expression of B7.1, B7.2, CD40 or ICAM-1 and with very little upregulation of MHCII [115]. The Th2 response was specific to DCs pulsed with SEA; it was not reproduced by untreated DCs. SEA therefore appears to induce DC activation in the absence of any conventional maturation.

It may also be possible to generate mature DCs that are no longer activated. DC activation is rapid. Export of MHCII complexes to the DC surface is observed within 2h of LPS stimulation [116] and IL-12 release can be detected within at 4h [117]. The process is also transient. Langenkamp *et al.* described the DC phenotype after activation as 'exhausted'. Their LPS stimulation triggered classical maturation in the DCs, but once that initial activity had passed, the capacity of the cells to secrete cytokines, particularly IL-12, was spent [118]. Reis e Sousa and colleagues reported a similar phenomenon, which they called 'paralysis' [119]. The timing of DC activation could be a significant factor in determining the outcome of T cell responses.

1.3 DC heterogeneity

1.3.1 Origins and Lineage

DCs are CD45+ leukocytes, originating in the bone marrow. This was neatly demonstrated in allogeneic bone marrow transplants: donor strain MHCII was expressed by Langerhans cells in the recipients' skin [120]. Early analysis of the DC phenotype noted the presence of certain monocyte and macrophage markers, including CD14 and F4/80 [19, 20]. The myeloid lineage of DCs was generally accepted, and the debate then was whether DCs represented an independent cell type or were merely veiled macrophages [121]. Steinman and colleagues demonstrated the shared ontogeny of DCs and macrophages in a series of colony forming assays: single cells gave rise to mixed populations [122]. Standard protocols for obtaining DCs *in vitro* involve their differentiation from monocyte precursors [91, 123], and the same appears to occur *in vivo* [124-126].

The paradigm of a myeloid, immunogenic DC is not always a valid one, however. Thymic DCs are thought to effect negative selection of developing T cells and contribute to tolerance rather than immunity [127, 128]. A significant number of these thymic DCs in mice display the CD8 surface marker typical of lymphoid cells [129]. The CD8 was shown to be endogenously produced, suggesting a lymphoid origin. This was supported by adoptive transfer experiments in which early T cell precursors, still capable of developing into B lymphocytes but not to erythroid or myeloid cells, formed both DC and T cell progeny when used to reconstitute an irradiated thymus [130, 131].

Although these key thymic reconstitution experiments were not clonal, formally leaving open the possibility that trace contamination by myeloid progenitors might account for the DCs that developed, the evidence for a lymphoid lineage is strong. DCs have been observed with B as well as T cell characteristics. Grouard *et al.* first described the human 'plasmacytoid T cell', which expresses the lymphoid marker CD4, resembles a plasma cell in appearance, and yet will readily differentiate into a DC when cultured in conditioned medium [132]. CD19 positive pro-B cells can

acquire a DC phenotype *in vitro* [133], and a subset of DCs found in murine lymphoid tissues has recently been identified by expression of the common B cell marker B220 [134, 135].

Further support for two distinct lineages of DCs was provided by the analysis of transcription factor mutants, which revealed differences in signalling requirements between DC subsets (table 1.5). PU.1 is an *ets*-family transcription factor expressed in early haematopoietic progenitors [136]; PU.1 knockout mice have CD8+ 'lymphoid' but not CD8- 'myeloid' DCs [137]. RelB is a component of NFκB

Transcription factor removed	Cells disrupted	Cells surviving	Reference
PU.1	CD8- myeloid DCs	CD8+ lymphoid DCs	[137]
RelB	All myeloid cells inc. most DCs	CD8+ DCs in spleen, thymic DCs	[138] [139]
Ikaros (dominant negative)	Lymphocytes, most DCs	Myeloid cells, LCs	[141, 142]
Ikaros (C terminal null)	B cells, NK cells, most DCs	Myeloid cells, LCs, T cells, CD8+ DCs	[143]

Table 1.5 Lymphoid and myeloid specific deficiencies in transcription factor knockout mice.

complexes and RelB null mice lack CD11c+ myeloid cells including the majority of DCs [138]. Wu *et al.* noted a residual population of splenic CD8+ DCs in these mice, and suggested that normal development of thymic DC would occur were the thymic medulla not so defective [139]. Conversely, Ikaros is a lymphoid specific transcription factor and a dominant negative mutant, Ik7, blocks the *in vitro* development of DC from human lymphoid precursors [140]. DC differentiation from peripheral monocytes was unaffected. The same dominant negative mutation in transgenic mice severely disrupts both lymphocyte and DC development. Myeloid

cell and epidermal LC populations survive intact [141, 142]. A null mutation at the C-terminus of *Ikaros*, specific to *Ikaros* and not affecting related genes, similarly depletes B and NK cells but some T cell development leaks through. Importantly, this is accompanied by the appearance of CD8+ DCs in the thymus [143].

These data strongly support the existence of lymphoid well as myeloid DCs, but the observations are not unambiguous. The extent of DC disruption in *Ikaros* deficient mice indicates either that all DCs other than LCs are lymphoid in origin, or that *Ikaros* is also involved in the development of some myeloid cells. A similar story emerges from an analysis of the cytokine dependence of different DC populations. Saunders *et al.* [144] have reported the unique ability of lymphoid DCs to develop in culture without GM-CSF, and they argue that the common use of GM-CSF as a support factor for DC growth *in vitro* may skew results towards apparently myeloid origins. The cytokine cocktail needed to replace GM-CSF was complex, however, and notably included IL-3 and IL-7. In the absence of these interleukins, GM-CSF regained its influence as a growth factor. The receptors for IL-3 and GM-CSF share a common β chain and may stimulate the same signalling networks [145]. Mice in which the GM-CSF gene is disrupted show little deficiency in DC number [146]. Again, this could reflect either the lymphoid origin of the majority of DCs, or merely the redundancy of cytokine function *in vivo*.

1.3.2 DC subsets

A discussion of DC lineage becomes largely academic without some implication of functional consequence. The evidence for an ontogenic influence on DC function *in vivo* is not yet clear. Thymic DCs are CD8+, are almost certainly lymphoid [130, 147] and mediate tolerance rather than immunity [148, 149], but the T cell outcome here may well reflect the developmental stage of the thymocytes rather than any inherent quality of the DCs [150]. CD8 expression is also a feature of a subset of DCs in the spleen, lymph nodes and certain non-lymphoid organs (fig 1.6) [129]. While it is clear that these cells can have unique functions [151, 152], it is unlikely

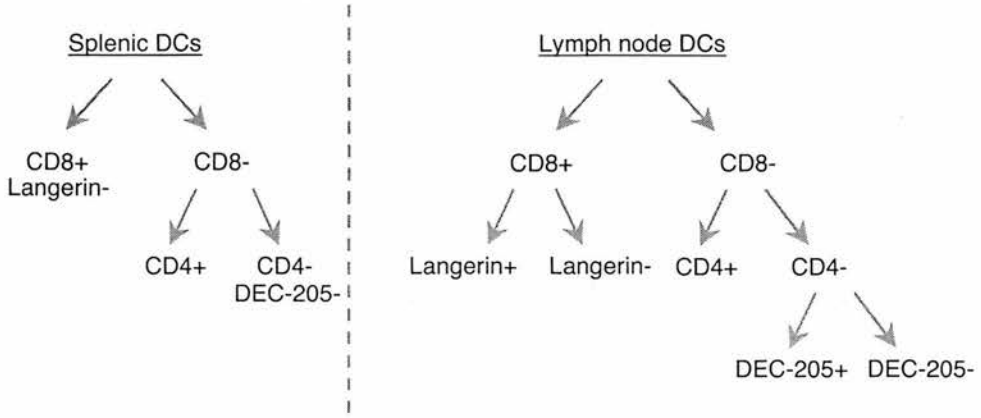

Figure 1.6 DC subsets of mouse lymphoid organs. Splenic DCs comprise three major subpopulations, defined by their expression of $CD8_{\alpha\alpha}$ and CD4. Lymph nodes contain the same three subsets with two additional populations, thought to arrive through the lymphatic system. One of these expresses low levels of CD8 but is Langerin positive and probably represents the mature form of epidermal Langerhans cells. The other is distinguished from CD8-CD4- double negative DCs by its expression of DEC-205, and is believed to be the mature version of tissue interstitial DCs. The arrows here denote only phenotypic divisions and do not indicate any lineage relationship.

that their CD8 expression is lineage specific. Splenic CD8+ DCs do not originate in the thymus: they are present as normal in athymic *nu/nu* mice [149]. They cannot then share the same lymphoid precursor as thymic DCs. Shortman and colleagues argue that equivalent lymphoid progenitors might exist in the bone marrow [153], but other researchers have demonstrated that splenic CD8+ DCs can derive from a highly purified CD8- DC population *in vivo* [154]. This suggested a shared and perhaps sequential myeloid lineage. Kamath *et al.* have used bromodeoxyuridine (BrdU) staining to assert the opposite [155], however, and the case is still undecided. Langerhans cells have been shown to upregulate CD8 expression on maturation, essentially converting from a CD8- to a CD8+ phenotype [156], and two other reports have described the emergence of CD8+ DCs from myeloid precursors [157, 158].

Although CD8 has disadvantages as a lineage marker, it does separate distinct functional populations. In keeping with the role of thymic DCs in central tolerance, CD8+ DCs in the spleen and lymph nodes were originally proposed to mediate peripheral tolerance [151]. Shortman and colleagues demonstrated suppression of both CD4 and CD8 T cell responses by CD8+ DCs. CD4 inhibition was associated with T cell apoptosis, perhaps due to the expression of FasL by CD8+ DCs [159]; CD8 suppression was due to failure of IL-2 induction [160, 161]. More recently, O'Connell *et al.* increased cardiac allograft survival by pretreating mice with donor strain CD8+ DCs [162], and Heath and Carbone have identified the APC responsible for cross-tolerance of the CD8+ T cell pool as a CD8+ DC [163, 164]. The same cell has been previously implicated in cross-priming [165], however, so factors other than DC subset are clearly involved.

In contrast to any tolerogenic role, several groups have reported the preferential induction of Th1 reactions by CD8+ DCs [152, 166, 167], and it is now widely accepted that CD8+ DCs are the dominant producers of IL-12 [168, 169]. Both conclusions serve to emphasise the influence of subset divisions on DC function, but otherwise reconciling tolerance with strong Th1 responses is difficult. The explanation may lie in the regulatory functions of Th1 cytokines such as IL-2 and IFNγ. IL-2 is a prototypic T cell growth factor, supporting the clonal expansion of activated T cells by both autocrine and paracrine routes [170]. IL-2 knockout mice are not immunodeficient, however, but instead display profound lymphocyte hyperplasia and symptoms of autoimmunity. Other cytokines appear to be able to compensate for its growth factor function, but its presence as an inhibitor is absolutely necessary to prevent anti-self responses [171]. IL-2 may potentiate Fas-mediated apoptosis [172-174], which is in keeping with the deletional tolerance seen among CD4 T cells responding to CD8+ DCs *in vitro* [159]. The Th1 cytokine IFNγ has also been reported to drive T cell apoptosis: cells lacking IFNγ are protected from activation-induced cell death [175]. T cell tolerance and Th1 proliferation may then be two sides of a fine balance, and in this situation, minor discrepancies in the cell purities and model antigens used in individual laboratories might be sufficient to generate quite different outcomes.

Distinct lineages and subsets of DCs exist in man as well as in mouse (reviewed in [169, 176]), although how exactly the populations correlate between species remains unclear [9]. Human DCs lack the CD8 marker seen in mice, but they do express CD4 [132] and the CD4+ CD3- CD11c- "plasmacytoid" DCs that emerge from cytokine stimulation of peripheral blood are probably lymphoid in origin [132, 177, 178]. Functionally, monocyte-derived, CD4- CD11c+ "DC1" cells respond to bacterial stimuli with a vigorous release of proinflammatory cytokines such as IL-12, TNFα and IL-6 [11, 179]. The plasmacytoid "DC2" cells and particularly their precursors, the pre-DC2s, are instead a major source of type 1 interferons [180, 181] and hence form an important anti-viral defence [182]. IFNα/β producing DCs have also been identified recently in mice [183]. The correlation between human and murine DCs is complicated by the difficulty of accessing equivalent tissues. Most human studies use peripheral blood, a scant resource in mice. Shortman and colleagues have recently begun a heroic investigation of the DC subsets in mouse blood (Shortman, 2002, NIH/NIAID meeting, Airlie, Virginia, USA) and, with support from rare descriptions of the DC populations in human spleen and tonsils [184-186], they maintain that a direct comparison of DCs in mice and men will reveal close similarities [9].

1.3.3 DC plasticity

The great attraction of DCs for immunotherapy is their ability to assess the nature and context of an antigen and to tailor the immune response appropriately. DC subsets make a substantial contribution to this flexibility, providing the total DC population with a variety of possible functions. Several mechanisms exist to target antigens to relevant DCs. Human, monocyte-derived 'DC1' cells express a different range of antigen recognition receptors from their plasmacytoid 'DC2' counterparts. DC1s display high levels of mannose receptors, favouring the uptake of polysaccharide antigens, while DC2 cells instead express a unique lectin molecule recognised by the antibody BDCA2 [132, 187]. In the mouse, separate DC populations have been defined by differential expression of the lectin DEC-205 [188-

190]. The TLR family of receptors also show subset specificity, with TLRs 2 and 4 predominantly expressed on DC1 cells and TLRs 7 and 9 restricted to DC2s [191, 192]. Such distinctions may extend to the level of antigen presentation: DC1 cells are particularly rich in CD1 molecules, enabling more efficient presentation of lipid moieties [193].

That said, the need to match each antigen with a particular DC seems surprisingly inefficient. It is improved by appropriate tissue localisation of the DC subsets. The best example of this is the dominance of tolerogenic lymphoid DCs in the thymus [190, 194], but other tissues also show characteristic immune responses and this too could be a result of polarised DC compositions. The LCs of the skin are classic examples of immunogenic DCs [20] and, correspondingly, antigen delivery by a subcutaneous route favours strong immunity [195]. DCs isolated from Peyer's Patches appear to be inherently skewed towards Th2 induction [196, 197], which may reflect the contribution of Peyer's Patches in providing access for intestinal antigens to the immune system. The liver is also an inherently tolerogenic organ [198, 199] and considerable effort has been made to identify and exploit the DC characteristics that contribute. Liver-derived DCs cultured with IL-3 and anti-CD40 were recently shown to induce regulatory T cells *in vitro* and prolong allograft survival *in vivo* [200].

Another unique quality of the liver is its cytokine microenvironment. Hepatocytes secrete TGFβ and IL-10 [201-203], both of which are reported to inhibit DC maturation and suppress T cell responses [204, 205]. The influence of these cytokines implies that the flexibility of DC function is not just a population effect but also a feature of individual cells. Indeed, Kalinski *et al.* have proposed a model in which equivalent myeloid DCs are matured and polarised by environmental signals, generating very different T cell outcomes [10]. The supporting evidence is strong. IFNγ has been shown to enhance DC production of IL-12 and skew responding T cells towards a Th1 phenotype, while prostaglandins suppressed IL-12 release and produced a Th2 response [10, 12]. Sato *et al.* stimulated DCs *in vitro* with GM-CSF, IL-3 and IL-12 and achieved a clear Th1 response; replacing the IL-12 with IL-4 instead promoted Th2 proliferation [206]. Human plasmacytoid DCs, which when

cultured with IL-3 will preferentially drive a Th2 reaction, are capable of eliciting IFNγ and IL-10 from T cells when stimulated with herpes simplex virus [182, 207]. Similarly, murine bone marrow-derived DCs can distinguish bacterial and parasitic antigens and stimulate type 1 and type 2 immunity accordingly [115, 208], even discriminating between the yeast and hyphae of the fungus Candida albicans [209].

The plasticity of DC function is not restricted to regulation of the Th1/Th2 balance. Kalinski's model also described 'type 3' DCs, generated in the presence of IL-10 [10]. IL-10 is an immunosuppressive cytokine, originally described as a product of polarised a Th2 clone [210] and now known to be released by a variety of cells including keratinocytes, B cells, macrophages and DCs [211, 212]. It downregulates the APC function of macrophages and DCs, reducing surface MHC and costimulation and inhibiting cytokine production [213-215]. Its effect on DCs is described as an inhibition of maturation: IL-10 is thought to prevent the upregulation of activation markers such as MHCII and B7.2, causing the DC to arrive in the lymph node and interact with T cells while still essentially immature [205, 216]. Although capable of forming surface MHC:peptide complexes [217], the low levels of CD40, B7.1 and B7.2 on these cells are thought to result in antigen presentation without adequate costimulation, leading to T cell tolerance [10, 216].

Other reagents have also been reported to inhibit DC maturation, including corticosteroids [218], vitamin D3 derivatives [219] and malaria-infected erythrocytes [30]. All appear to suppress T cell responses *in vitro*. The function of DCs is clearly influenced by their maturation state: immature DCs are thought to induce tolerance; mature DCs, immunity [220]. Their activation state may also be significant. In their characterisation of post-activation 'exhaustion', both Langenkamp *et al.* and Reis e Sousa *et al.* described the contrasting T cell reactions induced by DCs at different times after stimulation. The peak of activation was associated with a Th1 outcome; post-activation quiescence with Th2 [118, 119]. The importance of timing was reinforced recently by a similar description of kinetic regulation of NK cell activation [221].

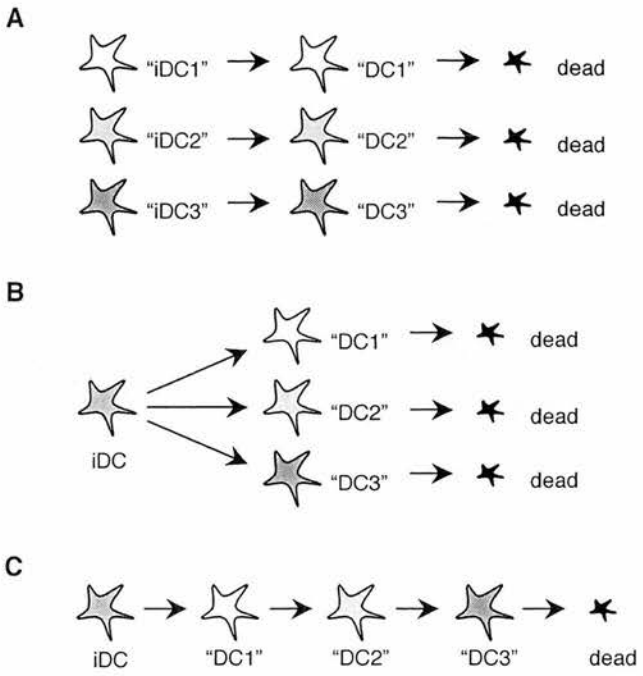

Figure 1.7 Models of DC differentiation. DCs show considerable flexibility of function. This could be a result of (A) the contribution of distinct subsets, (B) the influence of polarising signals and (C) the kinetics of DC activation.

The plasticity of DC function is apparent at several levels. Subsets, context and kinetics all contribute (fig 1.7). Despite the great debate between proponents of lineage and environment in the late 1990s [222], none of these influences are mutually exclusive. It may be, for instance, that the ontogeny of a DC dictates the extremes of its possible functions and the cytokine signals received during activation then determine where within that range the DC operates. The potentials of different DC populations may be overlapping but not necessarily identical. Indeed, Maldonado-Lopez *et al.* recently reported that the distinct functions of CD8+ and CD8- DCs can be partly but not wholly reversed by prior treatment with the cytokines IL-10 and IFNγ [12]. Similarly, Edwards *et al.* have described the ability of yeast extracts to elicit IL-10 release from all three splenic DC populations, CD8+CD4-, CD8-CD4+ and CD8-CD4-, while bacterial stimuli could only induce IL-12 secretion in two. The CD8-CD4+ DCs activated with *Mycobacterium* extract

were inhibited in their expression of IL-10 but could not make the full transition to IL-12 production [223].

1.4 DC : T cell interaction

The flexibility provided by a DC system of multiple subsets and individual plasticity enables immune responses to be moulded to fit their specific challenge. The translation of each DC phenotype into a qualitatively different immune reaction relies on a series of cell surface and soluble signalling events that occur during the initial DC:T cell interaction.

1.4.1 TCR engagement

As naïve T cells circulate through secondary lymphoid tissue, they form weak and transient attachments with waiting APCs through the interaction of leukocyte function antigen (LFA) -1 and CD2 on the T cell surface and ICAM-1,2 and 3 and LFA-3 on the DC. DC-SIGN is also important, contributing to the positioning of DCs in the T cell area [224] and reinforcing the early DC:T adhesion [33]. These events are thought to slow the passage of T cells through the lymph node, allowing TCRs time to sample the contents of MHC grooves on display. Signalling through the TCR further enhances T cell expression of adhesion molecules and induces a conformational change in LFA-1 that markedly increases its binding affinity for ICAM-3, stabilising cellular attachment [225].

This initial engagement of the TCR is followed within minutes by remodelling of both cell membranes to form a focus of tight contact, known as the immunological synapse [226, 227]. TCRs and costimulatory molecules become concentrated in a central supramolecular cluster (the cSMAC), surrounded by a ring of larger adhesion molecules such as CD2, LFA-1 and CD45, which are physically squeezed to the periphery (the pSMAC)[228]. Cytoskeletal rearrangements polarise the cells towards the synapse, bringing together receptor and signalling molecules and

enabling the efficient, directional secretion of cytokines [229]. The synapse facilitates the extended TCR signalling needed to commit the T cell to proliferation and IL-2 production. While effector or memory T cells can achieve this activation within 30 minutes, naïve cells can take between 6 and 30+ hours [230]. During this time, specific MHC:peptide complexes on the DC are thought to be repeatedly engaged and disengaged by a rapid turnover of TCRs on the T cell surface. In optimal conditions, 100 MHC:peptide complexes can bind and downregulate up to

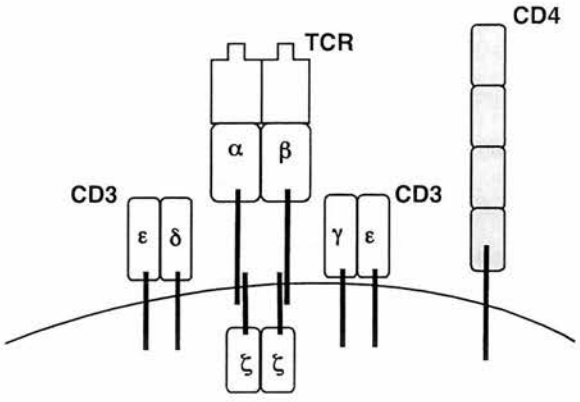

Figure 1.8 The TCR receptor. Peptide:MHC complexes on the DC are recognised and bound by the T cell receptor αβ heterodimer. Signalling occurs via ITAM motifs in the ε, δ, γ and especially ζ chains of CD3. The CD4 and CD8 coreceptors deliver src-family kinases to initiate the phosphorylation cascade.

20 000 TCRs within 5hours [231, 232]. This serial triggering causes an accumulation of downstream signalling events inside the T cell, eventually exceeding an activation threshold and inducing proliferation and effector cell differentiation [233].

The TCR clusters on the T cell surface with a CD3 complex, comprising γ,δ,ε and intracellular ζ chains (fig 1.8)[234]. The CD4 or CD8 coreceptor also associates, delivering the protein tyrosine kinase Lck which, together with a related kinase, Fyn, initiates the signalling cascade that translates TCR engagement into T cell activation. Lck and Fyn phosphorylate key sites in the immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the tails of the CD3 complex, leading to ZAP-70 recruitment and downstream activation of adaptor molecules (reviewed in [235]). This results in cytoskeletal reorganisation and altered gene expression, coordinated by transcription factors such as NF-AT, AP-1 and NFκB [236, 237].

1.4.2 Costimulation

In naïve cells, the coupling of TCR engagement to downstream signal transduction pathways is relatively inefficient [238]. Costimulation serves to stabilise and amplify the signals emanating from the TCR by recruiting kinases and adaptor proteins to the synapse [239]. The best characterised of the costimulatory interactions is that between the B7 molecules B7.1 and B7.2 on the DC and CD28 on the T cell. Antibodies against B7.2 inhibit a mixed leukocyte reaction and negate a primary antigen specific response [106, 240]. Other molecules also contribute. Activated DCs express 4-1BB ligand [241], which binds the 4-1BB receptor expressed on both CD4 and CD8 T cells and preferentially drives CD8 proliferation and IFNγ release [242], and the TNF family members CD40 and OX40L, which both have receptors on activated T cells [243, 244]. Costimulation is not restricted to contact dependent mechanisms. A recent report described IL-2 release by DCs, and this could act as a soluble mediator to initiate and support IL-2 production in responding T cells [109, 245].

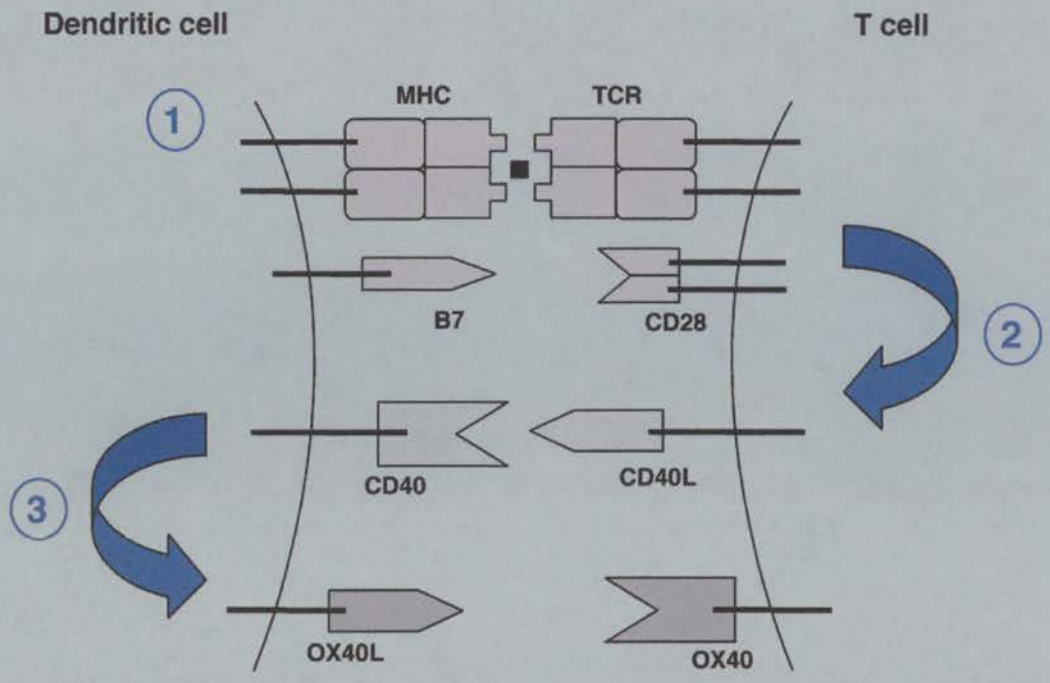

Figure 1.9 DC:T cell dialogue. Interaction between B7 on the DC and CD28 on the T cell augments peptide:MHC signalling through the TCR, and leads to T cell upregulation of CD40L. This binds CD40 and increases DC activation, inducing surface expression of OX40L. Signalling through OX40 increases T cell stimulation and encourages polarisation of the T cell response.

Costimulation is a two-way conversation. Activated T cells express TNF-related activation induced cytokine (TRANCE), which interacts with its receptor, receptor activator of NFκB (RANK), on the DC to potentiate cytokine release and prolong survival [246, 247]. Signalling via B7 can also regulate DC function: *in vivo* therapy with the high affinity B7 ligand CTLA-4-Ig was recently shown to regulate an immune response not just by blocking the binding of B7 to CD28 but also through its impact on DC metabolism [248, 249]. One of the T cell consequences of B7:CD28 communication is the enhanced expression of CD40 ligand, which then engages CD40 on the DC and elicits further DC activation, particularly increasing the production of cytokines such as IL-12 [243]. The additional stimulus given to the DCs also upregulates their expression of OX40L and so reinforces T cell costimulation through OX40L:OX40 interaction (fig 1.9)[244, 250].

1.5 T cell outcomes

1.5.1 Th1 vs Th2

Th1 and Th2 subsets were first identified by Mosmann and Coffman in 1986 [251]. They are defined by their distinct cytokine production and effector functions: Th1 cells secrete IL-2 and IFNγ and are potent activators of macrophages; Th2 cells release IL-4, IL-5 and IL-13 and support humoral immunity [252]. The two cytokine profiles are mutually antagonistic. IFNγ inhibits Th2 development; IL-4 and IL-10 both limit Th1 differentiation and dampen macrophage activity [253, 254]. The polarised phenotypes that result are echoed in CD8 T cells, B cells and even NK cells [221, 255-257]. Many factors influence the decision between Th1 and Th2 dominance of an immune response, and there is increasing evidence that DCs contribute by instructing the T cells at the moment of priming (reviewed in [168]). IL-12 and IL-4 are critical driving factors for Th1 and Th2 reactions respectively. DC production of IL-4 has been reported [209], although not yet been reproduced. In contrast, the ability of DCs to synthesise and release considerable quantities of IL-12 has been widely observed. Some IL-12 can be induced by strong activation signals such as LPS and other bacterial components [119, 258], but this response is potently enhanced by T cell interaction or anti-CD40 treatment [107, 259, 260]. Interestingly then, CD40 costimulation has recently been implicated in the development of a Th2 biased response. $CD40^{-/-}$ DCs were shown to be fully capable of supporting a Th1 reaction against bacterial antigens from *Propionibacterium acnes*, but were unable to elicit Th2 cytokines in response to an extract from *Schistosoma mansoni* [261]. The role of CD40-CD40L signalling appears dispensable for the activation of Th1 cells, but critical to Th2 differentiation.

Other costimulatory molecules are also thought to influence Th1 and Th2 responses. A dominance of B7.2 signalling over that of B7.1 has been reported to favour a Th2 reaction [262, 263]. OX40 costimulation is also associated with Th2 development, and anti-OX40 stimulation of naïve T cells *in vitro* induces IL-4 expression [264].

Lane and colleagues [250, 265] have argued that T cells receiving a strong signal through OX40 will respond by upregulating the chemokine receptor CXCR5, which acts as a homing receptor to guide them into B cell follicles. Once there, they provide cognate help for B cells, encouraging IL-4 release and class switching to the Th2 associated IgG1 isotype. OX40 costimulation is clearly also involved in the optimal function of Th1 cells, however, since an OX40-Ig fusion protein that blocks the T cell signal also prevents the development of inflammatory diseases such as inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE) [266, 267]. Lane's explanation is that the fusion protein also binds to OX40L expressed on vascular endothelium [268], and so prevents activated Th1 cells from entering the inflamed tissues to perform their effector functions [265]. It remains controversial. Normal T cells transferred into OX40L deficient mice will home to sites of inflammation; Chen *et al.* argue that the impaired hypersensitivity responses in these mice reflect the importance of OX40-OX40L interaction in the priming of Th1 cells [269].

The potential of OX40 signalling to direct Th2 reactions contrasts with the notion of Th2 as a default response that occurs in the absence of IL-12 [10]. Evidence suggests that a Th2 phenotype can occur by default: IL-12$^{-/-}$ DCs elicit IL-4 *in vivo* [152] and, in culture, 'exhausted' DCs that can no longer produce IL-12 will also trigger IL-4 release from responding T cells [118]. The lack of IL-12 is not always sufficient, however, and particularly in experiments using pathogen-derived antigens, a Th2 response also appears to involve positive induction. Jankovic *et al.* recently reported that, although IL-12$^{-/-}$ mice produce only limited IFNγ in response to antigen from *Toxoplasma gondii*, an intracellular parasite that normally generates powerful Th1 immunity, there was no compensatory increase in IL-4 expression [270]. This contrasted with results from MyD88 deficient animals which, when given the same challenge, displayed a strong Th2 response involving IL-4, IL-5, IL-10 and IL-13 [270]. These data suggest either that some component of the *T. gondii* extract signals to induce a Th2 response, which only becomes evident when the dominant Th1 response is completely inhibited, or that IL-12 is involved in Th2 as well as Th1 development [271]. Similar data had been provided by Kaisho *et al.*,

who demonstrated that activation of MyD88$^{-/-}$ DCs leads to a Th2 bias in a subsequent MLR and argued that this cannot be explained solely by the lack of pro-inflammatory cytokines made by these impaired DCs. MyD88 sufficient DCs derived from IL-12$^{-/-}$, IL-18$^{-/-}$ or double knockout mice failed to induce IFNγ in the MLR, but did not skew towards Th2 [272]. Kaisho *et al.* proposed that Th2 induction results from TLR signalling via the MyD88 independent pathway (fig 1.2). Others have argued for the existence of an independent family of PRRs, equivalent to the TLRs but specific for parasitic antigens, which are poised to direct the development of appropriate Th2 responses [273].

1.5.2 Immunity vs tolerance

The outcome of the DC influence on an immune response can be a Th1 or Th2 bias, or either immunity or tolerance [13, 14]. 'Tolerance' was originally used by Burnet to describe the negative complement to immunity necessary to protect against self destruction [274], but while Burnet referred only to the deletion of autoreactive T cell clones in the thymus, such central tolerance is now known to be incomplete. Self-reactive T cells with low affinity receptors or those specific for antigens not expressed in the thymus can escape into the periphery, and so additional mechanisms must act outside the thymus in order to prevent autoimmunity [82, 275]. This peripheral tolerance also serves to inhibit other unwanted immune responses, such as reactions to antigens within food or shed by commensal bacteria [276].

One form of peripheral tolerance is T cell ignorance: some self antigens are sequestered away from the immune system, hidden behind physical defences such as the blood-brain and blood-ocular barriers (BBB and BOB, respectively). These barriers are constructed from specialised vascular endothelia and close junctional complexes that together form a tight sieve to regulate the passage of macromolecules, pathogens and cells into the parenchyma of the brain or eye. They confer a status of 'immune privilege' on these tissues, reflecting the organs' importance for survival and their inability to regenerate [277-279]. The exclusion thought to be responsible for this 'privilege' was argued to prevent autoreactive T

cells from encountering their antigen, and to deny access to effector cells activated elsewhere. The implied risk was that any breach of the barrier, perhaps through traumatic insult, might allow ensuing autoimmunity to destroy whatever tissue the initial injury had left intact [280]. Limited lymphatic drainage and immune surveillance of the CNS and the eye does occur [281, 282], and other mechanisms contribute to the immune suppression in these sites. The tissues of the retina and cornea express FasL, causing Fas-mediated apoptosis of activated T cells *in situ* [283]. DCs populate the neural parenchyma only very sparsely [284, 285], and although resident microglia can upregulate MHCII expression upon activation [286], this is apparently in the absence of costimulatory molecules and so prohibits immunity [287, 288].

Antigen presentation without adequate costimulation is a classical method of tolerance induction [289, 290]. The T cell response is characterised by limited proliferation, a lack of cytokine production, particularly of IL-2, and the persistence of this hyporesponsiveness even after subsequent stimulation with fully competent APC. The phenomenon is known as T cell anergy, and it was first described in response to free peptide, presumably presented in a paracrine way by the MHCII+ human T cells to which it was given [291]. Other early demonstrations used fixed splenocytes [292] or purified MHCII molecules [293]. While a costimulation blockade is an effective method of disabling specific T cell responses *in vitro* [294], the inability to transcribe IL-2 is easily overcome by exogenous cytokine [295] and it seems a surprisingly fragile mechanism to be employed *in vivo*. Indeed, Matzinger [82] has argued that functional hyporesponsiveness can only be at best a temporary state that tolerised T cells pass through on their way to death, and at worst an *in vitro* artefact. Pape *et al.* have demonstrated the long-term survival of tolerised T cells *in vivo*, however, and they argue that neither immune deviation nor active suppression are responsible [296]. Whether purely experimental or also physiological, it appears that anergy can be an *in vivo* phenomenon.

1.5.3 Regulatory T cells

The contribution of active suppression to peripheral tolerance is currently the focus of renewed interest, with the recent publication of a wealth of new and largely *in vivo* data describing the existence of regulatory T cells [297]. Th1 and Th2 cells have long been reported to downregulate each other's function [254], so both could be described as 'regulatory' in certain situations. Indeed, immune deviation is a recognised form of T cell tolerance [298] and a Th2 balance has been shown to be protective in Th1-driven transplant rejection and autoimmune reactions [299, 300]. The use of immune deviation as a therapy is a risky one, however. Th2 reactions are not null responses and lupus is a crippling example of antibody-mediated pathology [301].

In contrast, one of the therapeutic attractions of the newly defined regulatory T cells is their potential to suppress both Th1 and Th2 responses [302]. The existence of a natural population of inhibitory cells was elegantly demonstrated by Powrie and colleagues, who reconstituted lymphocyte deficient SCID mice with T cells depleted of their $CD45RB^{lo}$ component and saw severe colitis develop 6-8 weeks later. The $CD45RB^{lo}$ cells alone did not induce colitis and, moreover, were able to prevent disease when the two populations were transferred together [303, 304]. The $CD45RB^{lo}$ population was functionally heterogeneous, containing memory and effector cells as well as those with regulatory action. Other groups reported a naturally occurring CD4+CD25+ regulatory T cell population [305, 306] and indeed CD25 was found to correlate more closely with inhibitory function in the colitis model than either of the CD45RB or CD38 markers used previously [307, 308]. The difficulty with all three of these molecules is that their expression is shared with activated CD4+ T cells irrespective of function, and to date the only definitive marker of regulatory T cells is their suppressive action [309].

The mechanisms by which regulatory T cells operate are uncertain. CD4+CD25+ "Tregs" are anergic in phenotype: they proliferate only poorly in response to stimulation [306]. They also appear to induce anergy in their target cells: they inhibit IL-2 expression in cocultured CD25- cells, and the constraint that this imposes on T

cell proliferation can be overcome by addition of exogenous IL-2 [310]. How exactly the suppression of IL-2 is achieved remains controversial. It was suggested that the constitutive expression of CD25 enables them to act as IL-2 'sinks', depriving otherwise responsive cells of access to sufficient IL-2 to initiate the autocrine amplification [170], but recently activated T cells expressing equivalent levels of CD25 do not suppress proliferation [310]. Regulatory T cells also require activation through their TCR in order to assume their inhibitory phenotype [310-312], although their targets do not need to share the same antigen specificity or even MHC restriction [310]. Physical contact between CD25+ Tregs and target cells is essential, as transwell separation eliminates suppression [310]. Although this suggests that soluble cytokines are not primarily responsible for CD25+ regulation, Nakamura *et al.* have proposed that surface bound TGFβ is critical [313]. They report that CD25+ T cells activated *in vitro* display a reactivity with an anti-TGFβ antibody that is not shared by their CD25- counterparts, and they argue that contradictory accounts of the inability of anti-TGFβ antibodies to abrogate suppression [310] are due to the failure of the antibodies to penetrate the tight contact between interacting cells. Perhaps conclusive data here is provided by TGFβ1$^{-/-}$ CD25+ T cells, which appear unhampered in their ability to suppress proliferation of cocultured cells (quoted as unpublished data in [314]). It is interesting to note, however, both that the TGFβ knockout mice from which these suppressive T cells were derived died prematurely from severe inflammatory disease [315], and that Powrie's original work with CD45RB^{lo} cells *in vivo* suggested a role for both TGFβ and IL-10. Neutralising antibodies abolished the protection against colitis that these cells otherwise provide [316].

Other descriptions of probably distinct regulatory T cell phenotypes have also cited the involvement of IL-10 and TGFβ. T regulatory type 1 cells, Tr1s, were originally generated *in vitro* by repetitive stimulation of CD4 T cells in the presence of IL-10 [317]. They were characterised by limited proliferation but considerable secretion of IL-10 and IL-5, without IL-2 or IL-4. Tr1 clones inhibit proliferation of naïve [317] and perhaps also memory CD4 T cells *in vitro* (quoted as unpublished data in [311]). *In vivo*, ova-specific Tr1 cells can prevent the colitis induced by CD45RB^{hi} cells in

SCID animals, although only when the mice are fed ovalbumin [317]. This implies that, like $CD45RB^{lo}$ or CD25+ regulatory T cells, Tr1 cells also require recent TCR triggering to become active, and that their effect is then non-specific. The suppression that these ova-specific Tr1 cells mediate acts against an immune response focussed on the self antigen or gut flora component that drives inflammatory bowel disease [318].

The action of Tr1 cells *in vitro* can be inhibited by either anti-IL-10 or anti-TGFβ, and most potently by the two antibodies in combination [317, 319, 320]. This distinguishes them from Th3 cells, a CD4 population proposed to suppress neighbouring cells by TGFβ alone [312, 321]. Th3 cells were first described as a result of experimental oral tolerance: EAE-prone SJL/J mice were fed low doses of myelin basic protein (MBP) and their mesenteric lymph nodes then used to generate T cell clones. Cells were identified which shared TCR specificity with potentially encephalitogenic Th1 clones, but which secreted TGFβ upon stimulation [322, 323]. These cells prevented the proliferation and cytokine production of Th1 clones *in vitro* and suppressed symptoms of EAE *in vivo*. A proportion of these 'Th3' clones also expressed IL-10 and IL-4, however, so their distinction from classical Th2 cells was not always clear. Indeed, Weiner has argued that the nature of a regulatory T cell is a product of the environment in which it functions, thus ensuring a degree and a mechanism of suppression pertinent to each situation [324]. Clean definitions of Th2, Th3 and Tr1 cells may only ever be artificial distinctions.

Can any cell become a suppressor, then, if stimulated in the right circumstances? 'Regulation' is not a unique feature of CD4 T cells. Suppressive versions of CD8 cells, NKT cells and B cells have all been reported [325-327]. They share common mechanisms: all rely at least partly on IL-10 as an effector molecule. An understanding of the signals required to initiate suppressive function in these effector cells would be a powerful therapeutic asset.

1.5.4 Tolerogenic DCs

The ability of DCs to influence the decision between tolerance and immunity was neatly illustrated by Hawiger *et al.*, who targeted antigen to undisturbed DCs *in vivo* by conjugating it to an anti-DEC-205 antibody. The hybrid antibody alone induced systemic tolerance; the same fusion protein given with an anti-CD40 antibody that stimulates the DCs, instead primed strong immunity [328]. This led to an understanding that immature DCs constitutively migrate into lymph nodes and establish and maintain tolerance to the self antigens that they present. As a consequence, the T cell reaction to an infection is focussed only on the foreign antigens and not against the self peptides that DCs inevitably also display [220]. This model is supported by two other reports of *in vivo* tolerance induction mediated by immature DCs [325, 329].

Activated DCs can also induce tolerance if directed by the environment or antigen. DCs harvested from the draining lymph nodes of the lung displayed high levels of surface MHCII, B7 and CD40 and yet elicited only minimal T cell proliferation *in vitro* [330]. They expressed IL-10, detected by both PCR and intracellular staining, and the limited T cell response was proposed to be a result of Tr1 induction. In contrast, DCs taken from mesenteric lymph nodes produced TGFβ and generated TGFβ-secreting T cells [330]. McGuirk *et al.* have described the ability of filamentous haemagglutinin (FHA) from *Bordatella pertussis*, a respiratory tract pathogen, to stimulate a DC phenotype that gives rise to Tr1 cells [112]. The authors offered two possible explanations: either the pathogen has evolved to exploit DCs to minimise the immune attack that it will face, or the host has deliberately employed tactics aimed to protect against the pathology of an unmitigated response. FHA deficient *B.pertussis* is cleared from the lung more rapidly than its wildtype equivalent. This is partly due to the loss of adhesion normally provided by FHA, but it also reflects an enhanced Th1 response from the host and is associated with increased damage to the airways (Mills, 2002, BSI congress, Harrogate, UK).

Evidence also suggests that the action of regulatory T cells can be mediated by DCs. DCs are not essential: activated CD4+CD25+ T cells will suppress CD8 cells in the

absence of APC [331]. A typical lymph node contains a prominent population of DCs, however, and both CD4 and CD8 regulatory T cells have been reported to act via induction of a tolerogenic DC phenotype [332, 333].

1.6 Summary

The importance of DCs in stimulating T cell responses gives them a powerful influence over the outcome of immune reactions. They combine information from their subset affiliation, the cytokine environment, the nature of the antigen and the strength of stimulation, and translate it into T cell stimulation through antigen presentation and costimulation. They can direct both Th1 or Th2 immunity and anergic or active tolerance. This functional flexibility provides DCs with considerable promise for therapeutic manipulation of the immune system.

1.7 Aims

This project originated in 1999, shortly after Kalinski *et al.* published their model of environmental instruction of DC function [10] and raised the possibility of manipulating DC phenotypes for therapeutic gain. With the hypothesis that *in vitro* conditioning of DCs could be used to direct immune responses *in vivo*, I began by activating DCs in the presence of different cytokines. These early experiments highlighted IL-10 as an immunosuppressive reagent capable of inducing a DC phenotype that elicited only limited T cell proliferation. This observation generated several questions:

- Is the influence of IL-10 on DCs an inhibition of maturation?
  (Chapter 3)

- Does the interaction with IL-10 treated DCs leave a lasting impact on the responding T cell?
  (Chapter 4)

- Can this *in vitro* effect also be seen if the DCs are administered *in vivo*? (Chapter 5)

- Do these DCs have any therapeutic benefit when applied to a disease model? (Chapter 6)

In attempting to address these issues, I hope this thesis offers some novel insight into the workings of dendritic cells and their potential to be exploited as immunotherapy.

Chapter 2 - Materials and Methods

2.1 Animals

All mice were maintained under specific pathogen free conditions in the animal facilities of the Faculties of Medicine and Science and Engineering at the University of Edinburgh. BALB/c and C57Bl/6 mice were either purchased from B&K Universal (Hull, UK) or bred in house; DO11.10 TCR transgenics (H-2A^d restricted, OVA peptide 323-339 specific) [334] were all bred in house. Mice were used at 6-12wk of age, and were sex and age matched within experiments. All experiments involving animals were performed under guidance from the appropriate Home Office personal and project licenses.

2.2 Media

Unless otherwise specified, medium was RPMI 1640 (Sigma-Aldrich, Poole, UK) supplemented with 5% foetal calf serum (FCS) (Labtech International, Andover, MA, USA), 2mM L-glutamine (Gibco BRL, Life Technologies, Paisley, UK), 50μM β-mercaptoethanol (2-ME) (BDH Merck, Poole, UK) and 50U/ml penicillin plus 50μg/ml streptomycin (Gibco).

Ex vivo cells were prepared and washed in Hanks Balanced Salt Solution (HBSS) (Sigma) with 2% FCS and antibiotics as above.

PBS was made in house by dissolving 137mM NaCl, 8.2mM $NaH_2PO_4.2H_20$, 2.7mM KCl and 1.5mM KH_2PO_4 in 0.2μm filtered dH_2O. Stocks were autoclaved to sterilise.

2.3 DC preparation

2.3.1 Splenic DCs by MACS purification

Spleens were removed from BALB/c mice and crushed between two glass slides to obtain a single cell suspension. Red blood cells were removed by incubation in red blood cell lysis buffer (Sigma) for 5min at room temperature. Cells were washed, resuspended at 10^8cells/900μl and mixed with 100μl of CD11c microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), as per the manufacturer's instructions. They were then incubated on ice for 15min, washed, resuspended and loaded onto pre-wet miniMACS MS columns in a miniMACS magnetic separator (Miltenyi). A maximum of $4x10^8$ cells were applied to a single column. The column was washed to dislodge unlabelled contaminants, then removed from the magnet and the positive fraction eluted using the supplied plunger.

2.3.2 Splenic DCs by plastic adherence

BALB/c spleens were removed and a single cell suspension prepared as above. Cells were resuspended in 10ml medium per spleen and distributed into 60mm, tissue culture grade petri dishes (Nalge Nunc International, Roskilde, Denmark) using 5ml cell suspension per plate. These were incubated at 37°C for 90min to enable macrophages and DCs to adhere. The plates were rinsed, using pre-warmed medium and gentle pipetting, until it could be seen that the majority of the non-adherent contaminants had been removed. The plates were given 5ml fresh culture medium, without GM-CSF, and returned to the incubator overnight. This long incubation allowed transiently adherent DCs to detach and they were harvested the following morning by more vigorous washing. Strongly adherent macrophages were left on the plates. Yields at this stage averaged $2\text{-}3x10^6$ cells per spleen and were 30-40% CD11c+MHCII+ DCs, as assessed by flow cytometry. The majority of the contaminants were CD19+ B cells.

To further enrich the DCs, cells collected after overnight adherence were depleted of B cells, T cells and granulocytes using Dynabead technology (Dynal, Oslo, Norway). Cells were resuspended at $2x10^7$/ml and incubated with uncongugated antibodies against CD19 (10μg/ml), Thy1.1 (5μg/ml) and Gr1 (5μg/ml) (all from Pharmingen, San Diego, CA, USA) for 30min at 4°C on a rotating mixer. Excess antibody was removed by washing in cold culture medium. Dynabeads were prepared for use by washing to remove the azide preservative and finally resuspended at $1x10^7$ beads/ml. Antibody-labelled cells were added to the bead suspension to give 6 beads per labelled cell and $4x10^8$ beads/ml, and the mixture incubated, rotating, for 30min at 4°C. Beads were then removed using a strong magnet (Dynal) and the unbound cells collected from the supernatant.

2.3.3 Bone marrow-derived DCs

DCs were grown from early bone marrow progenitors under the influence of GM-CSF, using a protocol based on that of Inaba *et al.* [335]. Bone marrow was flushed from the femurs and tibias of BALB/c mice using medium and gentle pressure from a 2.5ml syringe and a 25G needle. Cell clusters were dispersed by passage through a second 25G needle. Red blood cells were removed using lysis buffer (Sigma) and the remaining cells seeded into 24 well tissue culture plates at $3.75x10^5$cells/ml and 1ml per well. The culture medium was as normal, but using 10% FCS and without 2-ME. GM-CSF was added in the form of 5% supernatant from the transfected cell line X63-gmcsf [336], providing 10-15ng/ml GM-CSF. Levels of IL-10 in the supernatant were low (~90pg/ml) and those of IL-12 and TNFα were undetectable (Lynda Stuart, CIR, University of Edinburgh, personal communication). Cultures were washed at day 3 and day 6 to remove non-adherent granulocytes and lymphocytes. This involved swirling the plates before removing 1ml from each well and replacing it with fresh medium. On day 7, loosely adherent DCs were harvested by more vigorous washing, leaving firmly adherent macrophages attached to the plate. Preparations were 85-90% CD11c+, MHCII+ DCs (fig 3.1), and the remainder of the cells were predominantly Gr1+ granulocytes.

When bone marrow derived DCs (BMDCs) were used *in vivo*, the presence of FCS in their culture medium caused unwanted immune responses against serum antigens adsorbed onto the DC surface. The protocol was therefore modified to reduce the amount of FCS involved. Bone marrow suspensions were initially plated in 10% FCS, as above, to establish proliferation, but on days 3 and 6, this was replaced with 1% normal mouse serum (Harlan Sera Labs, Loughborough, UK). The GM-CSF supernatant, from a hybridoma grown in the presence of FCS, was identical in both cultures.

The bones from a single mouse typically released 2-3x10^7 BM precursors and so could be used to seed approximately two 24 well plates. If the culture was performed in FCS throughout, the expected yield from these two plates was 1-1.5x10^7 immature DCs. The yield was reduced in the presence of mouse serum, averaging 0.5-1x10^7 DCs. *In vivo* experiments therefore began with 2 plates of DCs for every 5 mice to be injected.

2.3.4 DC activation

Immature DCs were harvested from bone marrow cultures at day 7 and replated at 1x10^6cells/ml and 1ml per well in fresh 24 well plates, using DC culture medium with GM-CSF. LPS from *E.coli* 055:B5 (Sigma) was added at 0.1μg/ml. Cytokines were recombinant mouse proteins; IL-10 and IL-4 (both from R&D systems, Abingdon, UK) were used at 50ng/ml. Human prostaglandin E_2 (PGE_2) was a kind gift from Prof Rodney Kelly (MRC Centre for Reproductive Biology, University of Edinburgh). It had previously been shown to cross-react with mouse, and was used at 0.1μM.

2.3.5 Cytospins

5x10^4 DCs were resuspended in 200μl of medium or PBS containing 10% FCS, and spun onto Superfrost plus glass slides (BDH Merck, Poole, UK) using a Shandon

Cytospin 3 centrifuge (Shandon Inc, Pittsburgh, PA, USA) at 300rpm for 3min. The slides were dried in air for approximately 10min and then stained using Diff-Quick® reagents (Gamidor Ltd, Abingdon, UK). Staining comprised 2min in methanol, 2min in Diff-Quick I® (eosin) and 1min in Diff-Quick II® (haematoxylin). Slides were then rinsed in tap water, air dried and coverslips mounted in Pertex mounting medium (CellPath, Newtown, UK).

2.4 Other cell preparations

2.4.1 Unfractionated spleen and lymph nodes

Single cell suspensions of spleen were prepared in HBSS / 2% FCS, by crushing between glass slides and passage through a 25G needle. Red blood cells were removed with red blood cell lysis buffer (Sigma). Lymph nodes (popliteal, inguinal, brachial, axillary, superficial cervical, peri-aortic and mesenteric) were harvested, pooled and a single cell suspension prepared as for the spleens, but without red blood cell lysis. Both preparations were washed in medium and a viable cell count performed in 0.05% trypan blue (Sigma).

2.4.2 T cell purification

CD4+ T cells were purified from pooled spleen and lymph nodes by removing MHCII and CD8 positive contaminants with a MACS depletion column (Miltenyi). Cell preparations were stained with biotin-conjugated antibodies against MHCII (clone M5114; I-A specific; grown in house and used at 1:100) and CD8 (53-6.72; 1:100). Staining was for 15min on ice. The cells were washed and incubated with streptavidin-conjugated microbeads (Miltenyi) for 15min on ice, before being loaded onto a CS MACS column in a varioMACS magnet (Miltenyi). A single column was used for up to $2x10^8$ total cells. The column was washed and unbound cells

collected. They were routinely >80% CD3+ CD4+ (fig 5.2), and yields averaged $2x10^7$ cells per mouse.

2.4.3 Blood mononuclear cells

To monitor specific T cell expansion *in vivo* during DO11.10 adoptive transfer experiments (section 2.9) or to confirm reconstitution of irradiated bone marrow chimaeras (section 2.10), peripheral blood mononuclear cells were harvested and stained for analysis by flow cytometry. 50µl of tail blood was collected into an equal volume of heparin (1000U/ml; CP Pharmaceuticals, Wrexham, UK) and layered over 500µl Lympholyte^M (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada). After centrifugation at 2000rpm for 20min, mononuclear cells were harvested from the interface and washed before use.

2.5 Flow cytometry

Samples were analysed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA, USA), using a live cell gate set by forward and side scatter characteristics (see fig 3.5, for example).

2.5.1 Surface staining

Cells were taken from culture or directly after isolation and placed in a 96well, V-bottomed plate for staining. 1-$2x10^5$ cells were used routinely to collect 10 000 events on the flow cytometer. When the target population was small, as in the DO11.10 adoptive transfer experiments (section 2.9), $1x10^6$ cells were stained and 100 000 events collected. The cells were washed and resuspended in 100µl of antibody diluted in PBS supplemented with 2% FCS. Details of the specific antibodies and dilutions are listed in table 2.1. The cells were stained for 20min on

	Antibody	Clone	Source	Dilution	Isotype
DC markers					
	H-2D^d - biotin	19.19.1	In house	1:800	unknown
	I-A^d/I-E^d - FITC	2G9	Pharmingen	1:200	rIgG$_{2a}$
	CD11c - PE	HL3	Pharmingen	1:50	HsIgG$_1$
	CD11c - FITC	N418	In house	1:400	HsIgG$_1$
	B7.1 - PE	16-10A1	Pharmingen	1:100	HsIgG$_2$
	B7.2 - PE	GL1	Pharmingen	1:100	rIgG$_{2a}$
	CD40 - PE	3/23	Pharmingen	1:100	rIgG$_{2a}$
	ICAM-1 - PE	3E2	Pharmingen	1:200	HsIgG$_1$
T cell markers					
	CD4 - FITC	GK1.5	In house	1:250	rIgG$_{2b}$
	ova specific TCR - biotin	KJ-126	In house	1:100	mIgG$_{2a}$
	ova specific TCR - FITC	KJ-126	In house	1:400	mIgG$_{2a}$
	CD69 - PE	H1.2F3	Pharmingen	1:100	HsIgG$_1$
	CD25 - PE	7D4	In house	1:100	IgM
	CD45RB - FITC	16A	In house	1:100	rIgG$_{2a}$
	CD62L - PE	MEL-14	In house	1:100	rIgG$_{2a}$

	Antibody	Clone	Source	Dilution	Isotype
Other cells					
	Ly-6C (Gr1) - PE	RB6-8C5	Pharmingen	1:100	$rIgG_{2b}$
	CD19 - FITC	ID3	In house	1:100	$rIgG_{2a}$
	CD8 - biotin	53-6.72	In house	1:100	$rIgG_{2a}$
Cytokines					
	IL12p40/p7 - PE	C15.6	Pharmingen	1:100	$rIgG_1$
	IL-10 - PE	JES5-2A5	Pharmingen	1:100	$rIgG_1$
	IL-4 - PE	BVD6-24G2	Pharmingen	1:100	$rIgG_1$
	IFNγ - PE	XMG1.2	Pharmingen	1:100	$rIgG_1$
Secondary reagents					
	Streptavidin - PE		Calbiochem	1:1500	
Isotype controls					
	PE control	R35-95	Pharmingen		$rIgG_{2a}$
	PE control	A95-1	Pharmingen		$rIgG_{2b}$
	PE control	A19-3	Pharmingen		HsIgG
	FITC control	R35-95	Pharmingen		$rIgG_{2a}$

Table 2.1 Antibodies used in flow cytometry. Monoclonal primary antibodies were supplied by Pharmingen, Caltag Laboratories (Burlingame, CA, USA) or grown and conjugated in house (section 2.6). Streptavidin-PE was bought from Calbiochem (Nottingham, UK). Isotype controls from Pharmingen were used at the same concentration as the test antibody, and in house antibodies were matched with an irrelevant antibody of the same isotype at the same concentration. Abbreviations in isotype column: r, rat; Hs, hamster; m, mouse.

ice in the dark, washed and, if necessary, stained with a secondary antibody. Cells were resuspended in 200μl PBS / 2% FCS and analysed immediately.

To assess the surface phenotype of BMDCs, the membrane-impermeant dye ToPro3 (Molecular Probes, Leiden, The Netherlands) was included for the final 5min of staining at a final concentration of 1μM. This enabled the live cell gate to be set more accurately, using ToPro3 exclusion in addition to forward and side scatter characteristics.

2.5.2 Dead cell analysis

To quantify cell death, unstained cells were resuspended in 1μM ToPro3 immediately before acquisition. The analysis gate was set to exclude debris, and the proportion of ToPro3+ 'dead' cells calculated as a percentage of total cells within this population.

2.5.3 Antigen uptake assay

DCs were harvested after activation, washed and $2x10^5$ cells resuspended in 50μl PBS or FITC-dextran (2mg/ml in PBS; MW 70 000; Molecular Probes Inc, OR, USA) in a 96well, round bottomed plate. Cells were incubated at 37°C for 45min, to allow antigen internalisation, or on ice as a negative control. They were then flooded with 200μl ice cold PBS for 5min, washed twice and resuspended in 1% paraformaldehyde for analysis by flow cytometry.

2.5.4 Intracellular cytokine staining

BMDCs were activated with LPS or with LPS and IL-10 for 6h (as in section 2.3.5) in the presence of GolgiStop™ (Pharmingen) at a dilution of 1:1500, as

recommended by the manufacturer. Cells were stained with N418-FITC to detect surface CD11c expression, washed and then fixed and permeabilised by resuspending in Cytofix/Cytoperm™ reagent (Pharmingen) for 20min on ice. Cells were washed in permeabilisation buffer (provided in the Cytofix/Cytoperm™ kit) and stained with either anti-IL-12 or anti-IL-10 for 30min on ice. Subsequent washes were again performed in permeabilisation buffer and the cells then resuspended in PBS / 2% FCS for analysis by flow cytometry.

T cells from DC cocultures (section 2.7.2) were analysed for cytokine expression 7d after their original stimulation. Cells were harvested and replated in the presence of 50ng/ml phorbol 12-myristate 13-acetate (PMA), 500ng/ml ionomycin (both from Sigma) and GolgiStop™ (Pharmingen, as above) for 4 hours, before being stained with KJ-126-FITC and IFNγ, IL-4 or IL-10-PE, using the same protocol as described for DCs.

2.5.5 CFSE staining

T cell proliferation was visualised using CFSE (5, 6 carboxyfluoroscein diacetate succinimidyl ester) (Molecular Probes), a membrane permeant dye that binds covalently to cytoplasmic proteins and is distributed equally to each daughter cell upon division. The mean fluorescence therefore halves progressively as the cells divide [337]. Spleens and lymph node cells taken from mice immunised with ova peptide (section 2.9) were labelled with CFSE at 10nM for 8min at room temperature, washed and cultured in the presence of 0.5μg/ml ova peptide. Background fluorescence was determined for each sample 16-18h later, using flow cytometry. Cell division was assessed on day 3.

2.6 Antibody preparation

Supernatants from antibody-secreting hybridomas were collected and filtered to remove cells. Immunoglobulin was precipitated with ammonium sulphate, using 291g per litre of supernatant, and then dissolved in PBS and extensively dialysed against PBS. Antibodies were purified by binding to a protein G – sepharose column (Amersham, Buckinghamshire, UK) with elution at pH 2.8. Antibody concentration was estimated by measuring absorbance at 280nm.

Purified antibodies were conjugated to biotin by reacting each 1mg of protein with 75μg of succinimidyl-6 (biotinamido) hexanoate (EZ-Link™ NHS-LC-Biotin; Pierce, Rockford, IL, USA) in dimethyl formamide (Sigma). FITC conjugation was performed by reacting each 1mg of antibody with 50ng FITC Isomer I (Sigma) in a 0.15M sodium chloride / 50mM sodium carbonate buffer at pH9. Conjugated antibodies were then dialysed extensively against PBS to remove excess label. Antibodies were filter sterilised and stored frozen at –20°C.

2.7 Proliferation assays

2.7.1 Assessment of DC function

The stimulatory capacity of different DC populations was assessed by measuring the DO11.10 TCR transgenic (Tg) T cell proliferation elicited by antigen pulsed DCs. DCs were harvested after activation, washed and pulsed with graded doses of ova peptide (323-ISQAVHAAHAEINEAGR-339; Albachem Ltd., Edinburgh, UK) for 90min at 37°C. Pulsed DCs were washed and plated in 96well, round bottomed plates with CD4+ DO11.10 T cells at $1x10^4$ DCs plus $1x10^5$ T cells per 200μl well. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture (section 2.7.4).

2.7.2 Assessment of T cell function

To assess whether the initial DC interaction affected the subsequent function of the responding T cells, DCs were pulsed with ova peptide as above (section 2.7.1) and mixed with CD4+ DO11.10 T cells in a 48well plate, using $1x10^5$ DCs and $1x10^6$ T cells per 1ml well. After 3 days, all cells were recovered and centrifuged through Lympholyte^M to purify live T cells. These were returned to culture at $2x10^6$ cells/well in 1.5ml in a 24well plate, using medium supplemented with 1% supernatant from an IL-2 secreting hybridoma (a transfected cell line, X63-IL2 [338]). This supernatant was assessed by ELISA to contain 200ng/ml IL-2 when undiluted.

The T cells were collected again on day 7 of culture and either stained for cytokine detection (section 2.5.4) or plated with irradiated APC to measure their proliferation in response to restimulation. Spleens were taken from syngeneic BALB/c mice and irradiated whole with 4000rads from a caesium source irradiator. Single cell suspensions were made and plated at $5x10^5$ cells/well of a 96well, flat bottomed plate. $5x10^4$ T cells were added to each well in the presence of graded doses of ova peptide, to a final volume of 200μl. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture.

2.7.3 Spleen and lymph node restimulation assays

Spleens and lymph nodes from immunised mice were harvested and single cell suspensions plated at $6x10^5$ cells/well of a 96well, flat bottomed plate in 200μl final volume. Graded doses of ova peptide were added and proliferation measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture.

2.7.4 Cell harvesting and scintillation counting

Proliferation was measured by the addition of 0.5μCi of 35Ci/mmol [^{3}H]-thymidine (ICN, Basingstoke, UK) to each well, 16h before the end of culture. Plates were harvested onto filter mats (Printed Filtermat A; Wallac, Turku, Finland) using a 96well MachIIIM Tomtec harvester (Wallac). The mats were dried on a hot plate and a solid scintillation wax melted into them (Meltilex™ A; Wallac). When the wax had re-solidified, the mats were read using a Trilux 1450 Microbeta liquid scintillation and luminescence counter and software (Trilux, Arnsberg, Germany).

2.8 Polymerase chain reactions

2.8.1 RNA extraction

Total RNA was isolated using Trizol® reagent (Gibco) and a standard phenol-chloroform extraction. 1x10^6 DCs were resuspended in 500μl Trizol® and stored frozen at -80°C. Samples were thawed, incubated at room temperature for 5min and mixed with 100μl chloroform. After centrifugation, the upper aqueous phase was added to 250μl isopropanol, mixed and incubated at room temperature for 10min. Precipitated RNA was washed in 75% ethanol before being resuspended in 30μl nuclease-free water (Promega, Madison, WI, USA). RNA concentrations were estimated by measuring absorbance at 260nm.

2.8.2 RT-PCR

cDNA was synthesised using the Expand™ Reverse Transcriptase kit (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. Oligo(dT) primers were annealed to denatured RNA and reverse transcription then performed in a 20μl reaction containing 100mM DTT, 3.3mM each dNTP (Promega), 20U RNasin (Promega) and 0.05U Expand RT enzyme. Reactions were incubated for 60min at 42°C. cDNAs were stored frozen at -20°C.

Gene		Details
IL-12 p40		
	Sense primer	5'-ATGGCCATGTGGGAGCTGGAGAAAG-3'
	Antisense primer	5'-GTGGAGCAGCAGATGTGAGTGGCT-3'
	Programme	Denaturation - 94°C, 30s Annealing - 62°c, 30s Extension - 68°C, 30s 34 cycles
	Product	255bp
IL-12 p35		
	Sense primer	5'-GATCATGAAGACATCACACGG-3'
	Antisense primer	5'-AGAATGATCTGCTGATGGTTG -3'
	Programme	Denaturation - 94°C, 30s Annealing - 65°c, 30s Extension - 68°C, 30s 34 cycles
	Product	257bp
β-actin		
	Sense primer	5'-GCACCACACCTTCTACAATGAG-3'
	Antisense primer	5'-GTCTAGAGCAACAT AGCAC AGC-3'
	Programme	Denaturation - 94°C, 30s Annealing - 62°c, 30s Extension - 68°C, 30s 25 cycles
	Product	409bp

Table 2.2 Primers and programmes used for RT-PCR. See section 2.8.2.

Gene		Details
IL-12 p40		
	Sense primer	5'-ACATCTACCGAAGTCCAATGCA-3'
	Antisense primer	5'-GGAACACATGCCCACTTGCT-3'
	TaqMan probe	5'-CGTGCAAGCTCAGGATCGCTATTACAATTC-3'
IL-12 p35		
	Sense primer	5'-GTGAAAATGAAGCTCTGCATCCT-3'
	Antisense primer	5'-TCAGGCGGAGCTCAGATAGC-3'
	TaqMan probe	5'-CACGCCTTCAGCACCCGCG-3'
IL-10		
	Sense primer	5'-CCACAAAGCAGCCTTGCA-3'
	Antisense primer	5'-AGTAAGAGCAGGCAGCATAGCA-3'
	TaqMan probe	5'-AGAGCTCCATCATGCCTGGCTCAGC-3'

Table 2.3 Primers and probes used for real-time RT-PCR. See section 2.8.3.

PCR used *Taq* Supreme™ polymerase and buffers from Helena Biosciences (Sunderland, UK). 20μl reactions contained 2μl cDNA, 0.3mM each dNTP, 0.2mM each primer, 1.5mM $MgCl_2$ and 1U *Taq* Supreme™ enzyme. PCR was performed in a PTC-200 DNA Engine (MJ Research, Watertown, MA, USA). Primers and programmes are listed in table 2.2. PCR products were visualised on 2% agarose gels (ultraPURE agarose; Gibco) containing 0.5μg/ml ethidium bromide (Sigma).

2.8.3 Real time RT PCR

RNA samples were treated with DNase1 (Gibco). cDNA was synthesised using random hexamers and the TaqMan Reverse Transcription kit (Perkin-Elmer Applied Biosystems, Foster City, CA) as directed. Real-time RT-PCR was performed as described [339]. Relative quantification was done on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer) using pre-developed TaqMan reagents according to the manufacturer's instructions. Each reaction involved multiplex amplification of 18S rRNA to account for differences in the quantity or quality of RNA present. Thermal cycling conditions for all reactions were 2min at 50°C and 10min at 95°C, followed by 40 cycles of 2-step PCR consisting of 15s at 95°C and 1min at 60°C. All samples were amplified in triplicate. The threshold cycle C_t, which correlates inversely with target mRNA levels, was defined as the cycle number at which the emitted reporter fluorescence increased above a threshold level. For each sample, the amount of target mRNA was expressed as an *n*-fold difference relative to the amount of target mRNA expressed by unstimulated DC (designated the calibrator). The formula used to calculate these values is $2^{-\Delta\Delta Ct}$, where ΔC_t is determined by subtracting the average 18S rRNA C_t value from the average target C_t value. $\Delta\Delta C_t$ then involves subtraction of the ΔC_t calibrator value from the target ΔC_t value.

Primers and probes, to provide specific fluorescence, were designed by Dr Lynn Forsyth (CIR, University of Edinburgh) using Primer Express software (ABI Prism, Perkin-Elmer). They are listed in table 2.3.

2.9 Injections and immunisations

2.9.1 Adoptive transfer of DO11.10 cells

DO11.10 lymph node cells were filtered through parachute silk and injected i.v. into BALB/c recipients, using $5x10^6$ cells/mouse in 200µl PBS. The lymph nodes from one donor mouse usually yielded sufficient cells to transfer into 5 - 6 BALB/c hosts.

2.9.2 DC immunisation

Recipients of DO11.10 adoptive transfers were immunised 24h later with DCs coated with ova peptide. BMDCs were harvested at day 7 of culture and replated in medium alone, with LPS or with LPS and IL-10 for 6h (section 2.3.5). They were then washed and pulsed with 50µg/ml ova peptide for 90min at 37°C. Cells were washed again, counted and resuspended at $2.5x10^6$/ml in PBS, before being filtered through parachute silk. Mice were injected with $5x10^5$ DCs, either i.v. in 200µl or i.p. in 250µl (see section 5.6). Both routes were effective in inducing DO11.10 T cell expansion in the spleens and lymph nodes of the recipients.

2.9.3 CFA immunisation

Some mice receiving a DO11.10 adoptive transfer and subsequent DC immunisation were later rechallenged *in vivo* with ova peptide emulsified in complete Freund's adjuvant (CFA). 1mg/ml peptide was combined with an equal volume of CFA containing heat killed *Mycobacterium tuberculosis* H37RA (Sigma), vortexed and sonicated. 50µl volume was injected s.c. into each hind leg, thereby delivering 50µg ova peptide into each mouse, split between the two sites. Responses were assessed by harvesting the draining inguinal lymph nodes, processed as described in section 2.4.1.

2.10 Bone marrow chimaeras

Recipient BALB/c mice were lethally irradiated by exposure to 950rad over 45min, using a caesium source irradiator. 24h later they were reconstituted with pooled bone marrow cells from BALB/c and DO11.10 mice. A single cell suspension of each bone marrow population was prepared as in section 2.3.3 and depleted of Thy1+ T cells by staining with a biotinylated anti-Thy1 antibody (T24 biotin, produced in house), streptavidin-conjugated microbeads and subsequent removal on a MACS depletion column (Miltenyi, section 2.4.2). This aimed to prevent graft-versus-host disease in the recipient mice. BALB/c and DO11.10 cells were then combined at a ratio of 9:1, filtered through parachute silk and $5x10^6$ injected i.v. into each irradiated host. The chimaeras were left 8 weeks before use, to allow reconstitution of the immune system. This was confirmed by a normal ratio of B to T cells in the peripheral blood, measured by flow cytometry (section 2.4.3).

2.11 EAE induction

2.11.1 Immunisation with MOG_{35-55}

C57Bl/6 mice were immunised with 100μg MOG_{35-55} peptide (Advanced Biotechnology Centre, Imperial College, London, UK) emulsified in CFA, given as two 50μl s.c. injections, one into each hind leg (see section 2.9.3). Mice also received 200ng of pertussis toxin i.p. in 500μl PBS on the same day and two days later.

2.11.2 Clinical scoring

Clinical signs of EAE were assessed daily using a discrete 0-6 scoring system [327]. 0, no signs; 1, flaccid tail; 2, impaired righting reflex or unstable gait; 3, partial hind

limb paralysis; 4, total hind limb paralysis; 5, hind limb paralysis with partial fore limb paralysis; 6, moribund or dead. Mice at grade 5 for two consecutive days or at grade 6 were humanely killed and their scores recorded as grade 6 throughout the remainder of the experiment.

2.11.3 Cytokine ELISA

Spleen and inguinal lymph node cells of mice recovered from EAE, 30d after immunisation, were cultured with MOG_{35-55} peptide and their cytokine production measured by a cell-based ELISA protocol [340]. Single cell suspensions were plated at $6x10^5$ cells/well in a 96well, flat bottomed plate. Graded doses of MOG_{35-55} peptide were added to a final volume of 200µl, using X-Vivo 15 serum free culture medium (BioWhittaker, Maidenhead, UK) supplemented with 2mM L-glutamine and 50µM 2-ME. After 48h, 100µl of cells were transferred to MaxiSorb microtiter plates (Nalge Nunc), precoated with anti-cytokine antibody and blocked in PBS / 1% bovine serum albumin (ELISA grade; Sigma). The cells were cultured at 37°C for a further 24h before the plates were washed, incubated with biotinylated detection antibodies for 1h at room temperature, extravidin-peroxidase (Sigma) for 30min and then developed with 10% w/v tetramethylbenzidine (TMB) substrate (Sigma) diluted in substrate buffer (0.05M sodium phosphate, 0.025M sodium citrate, pH5) with 0.03% hydrogen peroxide. Reactions were stopped with 2M H_2SO_4, and plates were read at 450nm using a MultiSkan Ascent reader and software (Thermo Labsystems, Vantaa, Finland). Paired antibodies and recombinant cytokine standards were bought from Pharmingen, and are listed in table 2.4.

Cytokine detected	Capture Ab	Standard	Detection Ab
IL-2	JES6-1A12 2μg/ml	Recombinant mouse IL-2, beginning at 5ng/ml	JES6-5H4 0.5μg/ml
IL-4	11B11 2μg/ml	Recombinant mouse IL-4, beginning at 5ng/ml	BVD6-24G2 0.5μg/ml
IL-10	JES5-2A5 2μg/ml	Recombinant mouse IL-10, beginning at 100ng/ml	SXC-1 0.5μg/ml
IFNγ	R4-6A2 2μg/ml	Recombinant mouse IFNγ, beginning at 100ng/ml	XMG1.2 0.5μg/ml

Table 2.4 Antibodies and cytokines used for cell-based ELISA assays. See section 2.11.3.

2.12 Statistics

Statistics were calculated using GraphPad Prism® (version 3.0, GraphPad software, San Diego, CA, USA, www.graphpad.com), following the recommendations made by this programme. Results were considered significant if $P<0.05$.

Chapter 3 - DC activation in the presence of IL-10

3.1 Introduction

DCs can elicit very different immune responses. Langerhans cells classically promote the development of strong immunity and contact hypersensitivity [89, 341], while thymic DCs establish central tolerance by deleting responding T cells [150, 342]. The factors that control such diverse functions remain the matter of some debate [222]. Shortman and colleagues originally argued that thymic DCs develop from lymphoid precursors distinct from the myeloid cells thought to give rise to other DC types [130]. A subset of DCs in spleen and lymph node were also identified as lymphoid in origin, and it was suggested that these too might tolerise T cells by Fas-mediated deletion [159, 189]. The story has been complicated by the description of many more DC subsets, but the idea that the function of a DC is fixed by its lineage has persisted. Moser [152] and others [167] have reported that only CD8α+ DCs are able to release the IL-12 that can drive IFNγ production and a Th1 response. Pulendran *et al.* described differences in DC localisation and antigen uptake that correlated with lineage [188].

Intuitively, it is hard to accept that the performance of a DC is determined solely by its ontogeny. This seems unfeasibly rigid in an immune system that must respond appropriately to innumerable different insults. There is a growing body of evidence that DCs are plastic, capable of reading external signals and tailoring the immune response appropriately. Cytokines can polarise DC function: IFNγ enhances DC production of IL-12, thereby skewing the T cell response towards a Th1 phenotype, while prostaglandins suppress IL-12 release and allow the development of Th2 cells [343, 344]. Different pathogens are also influential. DCs can distinguish between bacterial and parasitic antigens and stimulate type 1 or type 2 immunity accordingly, even discriminating between the yeast and hyphae of the fungus *Candida albicans* [115, 208, 209].

One of the attractions of DC plasticity is that it implies that DCs are open to modulation, and so could be used to manipulate the immune system. If the presence of different cytokines or other agents during DC activation can determine their function, these cells have considerable therapeutic promise. With the aim of generating tolerogenic DCs for use *in vivo*, I began by stimulating DCs with LPS in the context of IL-10, IL-4 or PGE_2. This chapter describes the different phenotypes that were achieved. While PGE_2 reduced the ability of DCs to stimulate T cells, IL-4 surprisingly enhanced it. The effect of IL-10 was most pronounced, however. DCs activated in the presence of IL-10 showed an early upregulation of MHC and costimulatory molecules, followed by rapid downregulation. The induction of these surface markers occurred in the absence of full IL-12 expression and without detectable IL-10 production. IL-10 appears to act not by holding DCs in immaturity, but rather by dictating the kinetics and quality of their activation.

3.2 Approach

Initial experiments identified bone marrow-derived dendritic cells as a source of DCs open to manipulation. When the cells were harvested at day 7 of culture, they had a largely immature phenotype, with little MHCII or B7.2 on their surface. These cells were replated into medium containing both LPS and different cytokines, to drive DC maturation in the presence of putative polarising signals. The DCs were harvested at various times afterwards and either phenotyped by flow cytometry and PCR or assessed functionally in T cell proliferation assays.

3.3 Results

3.3.1 Dendritic cell sources

In order to obtain a population of dendritic cells to study, splenic DCs were first compared with those grown *in vitro* from early bone marrow progenitors (fig 3.1).

Initial attempts to purify splenic DCs used MACS technology to positively select CD11c+ cells. Yields were unpredictable (0.5 to 6 $x10^6$ DC/spleen), however, and their viability predictably poor (typically <50%). As an alternative, plastic adherence was used to enrich DCs to ~30-40% of splenocytes. The majority of contaminants were B cells, and subsequent depletions of B cells, T cells and granulocytes left a highly purifed DC population (~95% CD11c+ MHCII+; fig 3.1C). Cell death was still considerable (see fig 3.1B), but viable yields averaged $5x10^5$ DC/spleen. Given that DCs are thought to comprise 1-1.5% of mouse spleen [284, 345], this suggests reasonably efficient recovery.

The major disadvantage of these splenic DCs was that, probably due to both their origin in secondary lymphoid tissue and the protracted isolation procedure, the cells had an extremely mature phenotype: staining for both MHCII and B7.2 was intense (fig 3.1C, D). Mature DCs are reported to be resistant to polarising signals [205, 346], and so in order to manipulate DCs by maturing them in the presence of different cytokines, a source of immature cells was needed. Bone marrow-derived DCs (BMDCs) offered a more malleable population. Harvesting BMDCs at day 7 of culture yielded cells that were predominantly alive (fig 3.1F), were 85-90% pure (fig 3.1G) and, importantly, expressed only low levels of MHCII and B7.2 on their surface (fig 3.1G, H). The numbers of DCs generated in this way were also several fold higher than those isolated from spleen. The femurs from a single mouse typically released ~$2x10^7$ bone marrow precursors, which could produce 1 - $1.5x10^7$ immature DCs by day 7.

While the extended culture period required to obtain BMDCs may make them less physiologically relevant than *ex vivo* splenic DCs, equivalent protocols exist for growing DCs from myeloid precursors in human blood [25] [347]. BMDCs therefore have a strong therapeutic relevance. For this reason, and because they could provide access to plentiful immature DCs, BMDC cultures were used for all subsequent experiments.

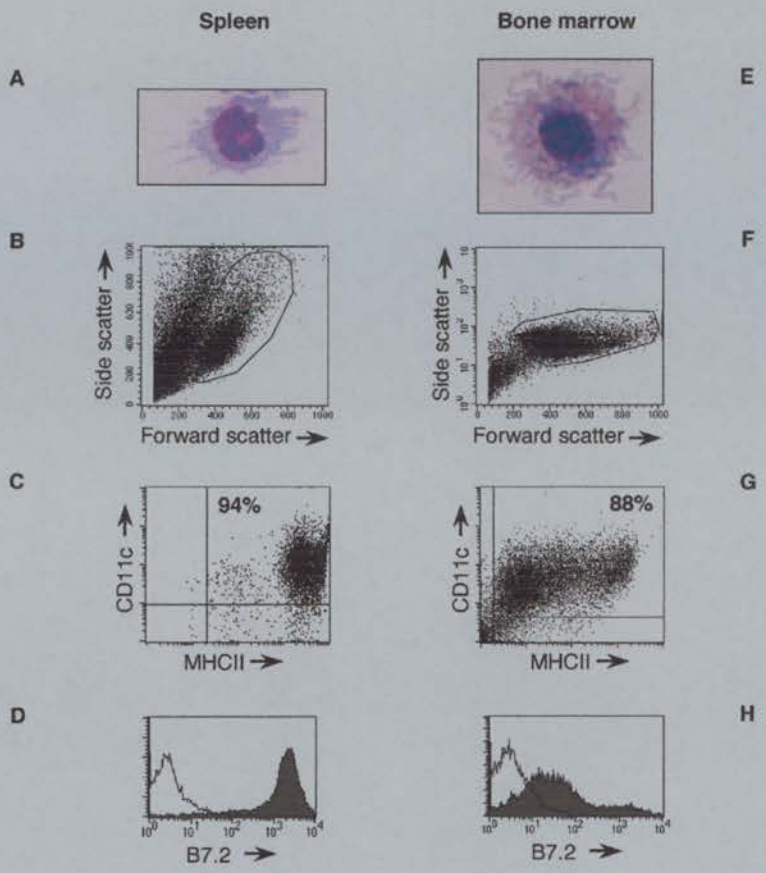

Figure 3.1 Comparison of spleen- and bone marrow-derived DCs. DCs were either purified from mouse spleen using plastic adherence and depletion of contaminating lymphocytes and granulocytes, or grown from bone marrow precursors in the presence of GM-CSF and harvested at day 7 of culture. This figure depicts their phenotype immediately after isolation or harvest. (A&E) Photographs of cytospins stained with haematoxylin and eosin. Magnification, x1000. (B&F) Forward and side scatter profiles, assessed by flow cytometry. Note the different scales, linear vs log, on the side scatter axes. The live cell gate used for subsequent analysis is shown. (C&G) Double staining for CD11c and MHCII, used as an estimate of dendritic cell purity. The numbers give the percentage of live cells falling in the top right quadrant. (D&H) B7.2 expression. Open peaks indicate isotype controls. Data in all panels are representative of at least five separate experiments.

3.3.2 DC maturation

The maturation process of dendritic cells was mimicked *in vitro* by stimulating them with LPS from *E. coli* bacteria, an archetypal activation signal. Murine bone marrow derived DC are notoriously prone to spontaneous maturation and indeed the unstimulated population already displayed a reasonable level of MHC and costimulation on their surface. This probably reflects both the age of the cells and the disruption involved in their replating. 24 hours' culture in LPS, however, further increased expression of both MHCI and MHCII and the costimulators B7.1, B7.2, CD40 and ICAM-1 (fig 3.2A). When the DC were coated with peptide and cocultured with specific T cells, this LPS-induced "maturation" enhanced T cell proliferation (fig 3.2B).

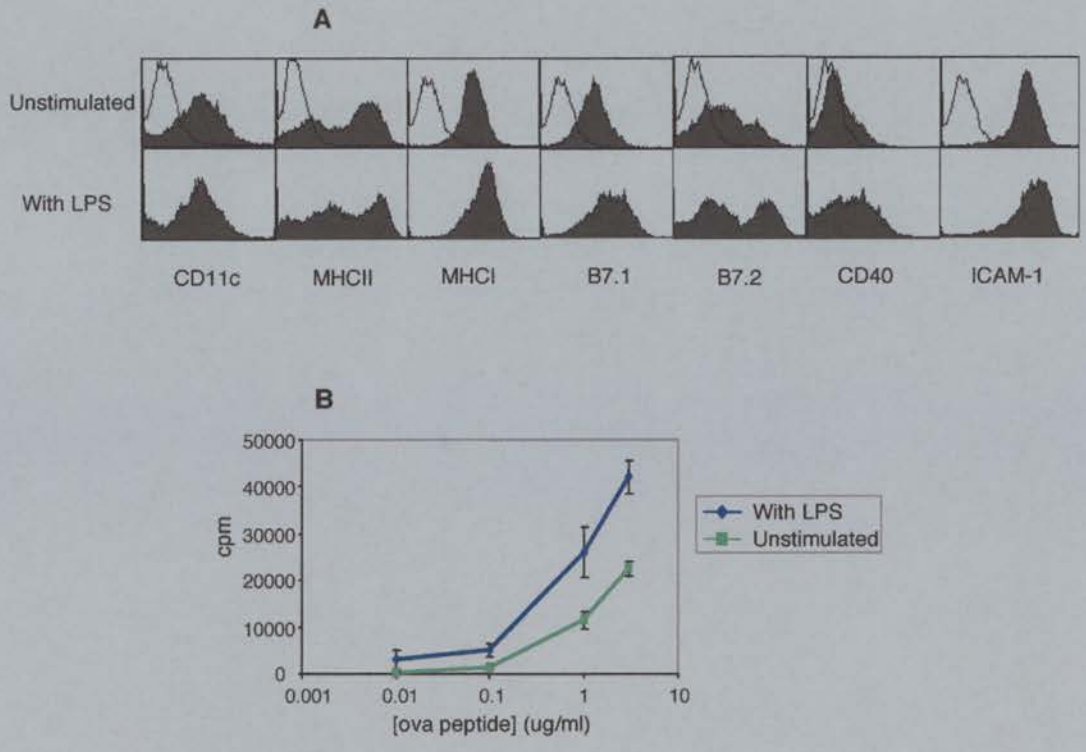

Figure 3.2 Dendritic cell maturation. DCs were harvested at day 7 of culture and replated in medium alone (unstimulated) or in the presence of LPS (0.1μg/ml). (A) Surface marker expression 24h after stimulation, measured by flow cytometry. Open peaks represent isotype controls. (B) T cell stimulatory capacity, also at 24h. DCs were pulsed with ova peptide and mixed with CD4+ cells purified from DO11.10 TCR Tg mice. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate cultures ± SEM. Both panels are representative of at least three different experiments.

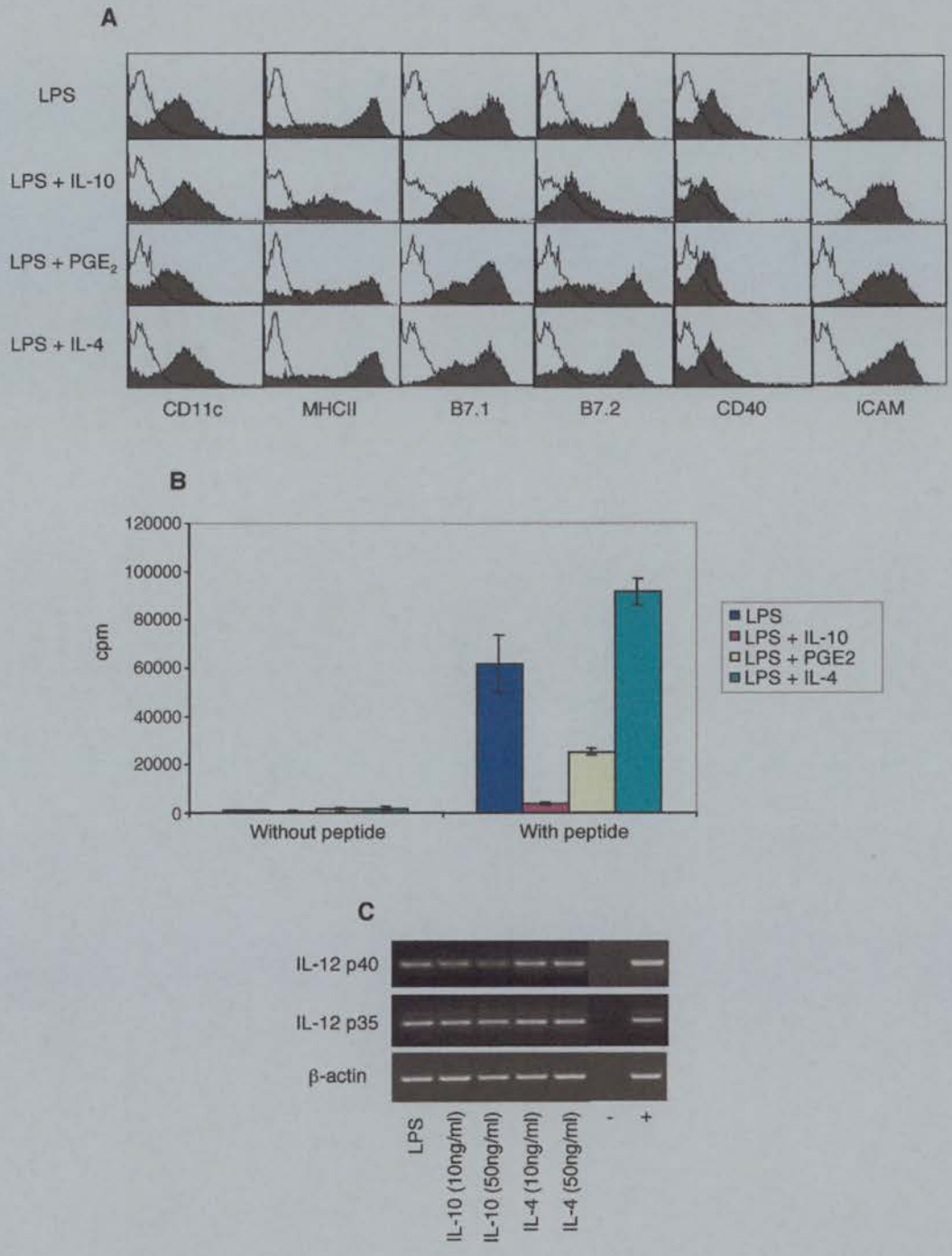

Figure 3.3 DCs are responsive to environmental signals. DCs were harvested at day 7 of culture and stimulated with LPS for 24h either alone or in combination with IL-10 (50ng/ml), PGE_2 (0.1μM) or IL-4(50ng/ml). (A) Surface phenotype, assessed by flow cytometry. Open peaks indicate isotype controls. (B) T cell proliferation. DCs were pulsed with ova peptide at 1μg/ml and mixed with DO11.10 T cells. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data represents the mean of triplicate cultures ± SEM. (C) DC IL-12 expression, assessed by RT-PCR. -, H_20 control. All panels are representative of three independent experiments.

3.3.3 Polarisation by cytokines

E. coli LPS alone generates strongly immunogenic DCs that secrete IL-12 and favour a Th1 response [7, 348]. To elicit a contrasting phenotype, DCs were activated with LPS in the presence of IL-10, PGE_2 or IL-4.

The data here support the argument for DC plasticity: different phenotypes and, at least in terms of T cell stimulation, different functions were achieved from a single DC prep (fig 3.3). The influence of the individual cytokines was interesting. At this 24h timepoint, IL-10 caused a profound downregulation of both MHC and costimulator expression and consequent T cell proliferation. PGE_2 suggested a similar effect, but its downregulation was less severe and this discrepancy could not be overcome by increasing its concentration. The action of the two cytokines was clearly not identical. Most surprising was the impact of IL-4. IL-4 is a model Th2 cytokine and I expected it to suppress the immunogenicity and the Th1 inducing capacity of DCs. In contrast, its effect was a slight enhancement of LPS-induced activation, seen in terms of the surface phenotype of the DCs (fig 3.3A), the proliferation of the responding T cells (fig 3.3B) and the DC expression of IL-12 (fig 3.3C).

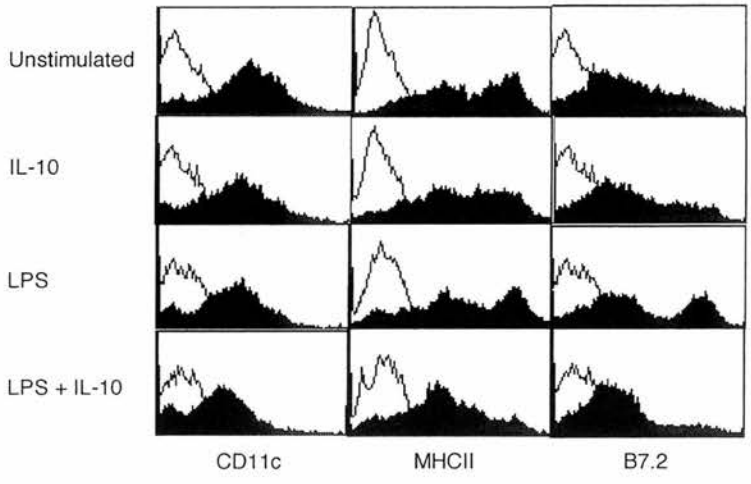

Figure 3.4 IL-10 alone is not a DC activation signal. DCs were replated at day 7 and cultured for 24h with or without LPS, in the presence or absence of IL-10. Their surface phenotype was assessed by flow cytometry. Open peaks represent isotype controls. Data is representative of three separate experiments.

3.3.4 Activation in the presence of IL-10

While the synergy between LPS and IL-4 was intriguing, I wanted to obtain a DC phenotype that could be used to regulate T cell responses. IL-10 appeared the best candidate. The marked suppression of surface MHC and costimulation and of T cell proliferation seen in figure 3.3 has been understood as an inhibition of maturation: the presence of IL-10 negates the effect of LPS and prevents upregulation of activation markers [205, 349]. The impact of IL-10 appears more profound, however. After 24 hours' stimulation, IL-10 suppressed both MHCII and B7.2 expression to a level not equal to, but consistently below that of the unstimulated control (compare figs 3.2 and 3.3; also evident in fig 3.4). While IL-10 alone seems to do little to disturb immature cells – it is not itself an activation signal (fig 3.4) – the downregulation caused by LPS and IL-10 together suggests the DCs have made some kind of active response to the combined signal. In support, at just 6 hours post-stimulation, both IL-10 treated and LPS alone populations were activated: their expression of MHCII and B7.2 was high, and higher than that seen at 24h with LPS alone (fig 3.5). This is exciting first because it demonstrates that LPS-driven activation is transient, with MHCII and B7.2 up- and then down-regulated within 24h, and second because it demonstrates that activation does occur in the presence of IL-10. The regulatory function of this cytokine is not a complete inhibition of maturation.

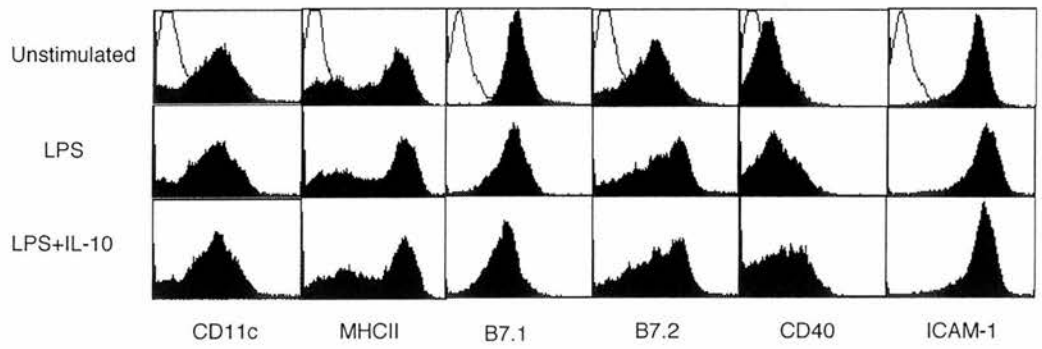

Figure 3.5 IL-10 treated DCs respond to stimulation. DCs were replated and stimulated with LPS, in the presence or absence of IL-10. This figure shows their surface phenotype 6h after activation; compare with figure 3.4, taken at 24h. Open peaks indicate isotype controls. Data is representative of five different experiments.

3.3.5 Downregulation of antigen acquisition

To confirm the maturation status of IL-10 treated DCs, their ability to internalise a fluorescent antigen was assessed. The switch from antigen uptake to antigen presentation is, at least *in vitro*, a hallmark of DC maturation [4, 350]; this allows antigen uptake to be used as a convenient measure of immaturity. Unstimulated, 'immature' DCs contained a population of cells that actively internalised FITC-dextran and became brightly fluorescent. Cells that had been matured in LPS for 24h acquired less antigen: in the experiment shown, the mean fluorescence of this population fell from 200 to 66 (fig 3.6). DCs stimulated with LPS and IL-10 also showed reduced antigen uptake, their fluorescence matching that of the mature, LPS-treated DCs rather than immature cells.

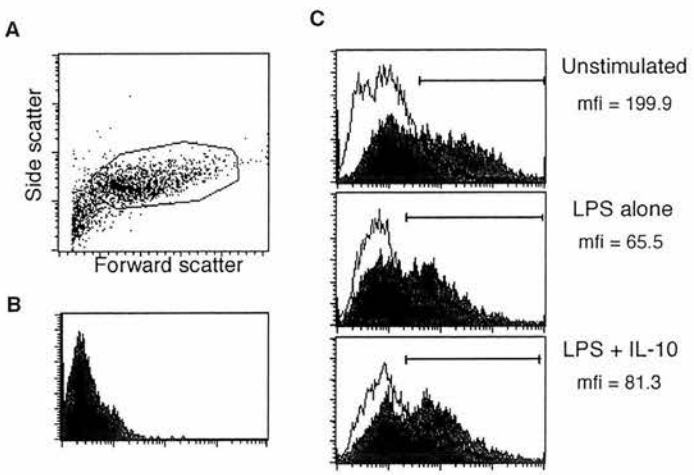

Figure 3.6 IL-10 treated DCs downregulate antigen uptake. DCs were given LPS and IL-10 for 24h before being washed and pulsed with FITC-dextran. Internalisation was assessed by flow cytometry. (A) Forward and side scatter plot showing the live cell gate used for analysis. (B) Background autofluorescence, measured on unstimulated cells pulsed with PBS alone. (C) Antigen uptake assay. Open peaks indicate background uptake at 0°C, filled peaks specific uptake at 37°C. Numbers give the mean fluorescent intensity for cells within the marker shown. Data is representative of three independent experiments.

3.3.6 Acceleration of activation kinetics

The presence of IL-10 during DC activation does not prevent the early upregulation of surface markers, nor the loss of an immature phenotype, but it does suppress the levels of MHC and costimulation seen 24h after stimulation. Intrigued by the transience of activation, the expression of these markers was followed over time. The results are shown in figure 3.7. They reveal a clear wave of activation, with expression increasing, peaking and falling again within the 24h period. The variation was most striking for B7.2 but also occurred for MHCII and, to a lesser degree, for B7.1. Expression of all three markers peaked at 6-12h with LPS alone and was beginning to fade by 24h. Cells stimulated with LPS plus IL-10 achieved equivalent levels of expression, but the peak occurred earlier, around 3-6h, and downregulation was much more advanced by 24h. Throughout the timecourse, the expression of the DC marker CD11c was stable and comparable for both populations of cells. Thus, in terms of surface phenotype, the effect of IL-10 is to drive LPS activated DCs more quickly into and out of activation. Its influence is kinetic.

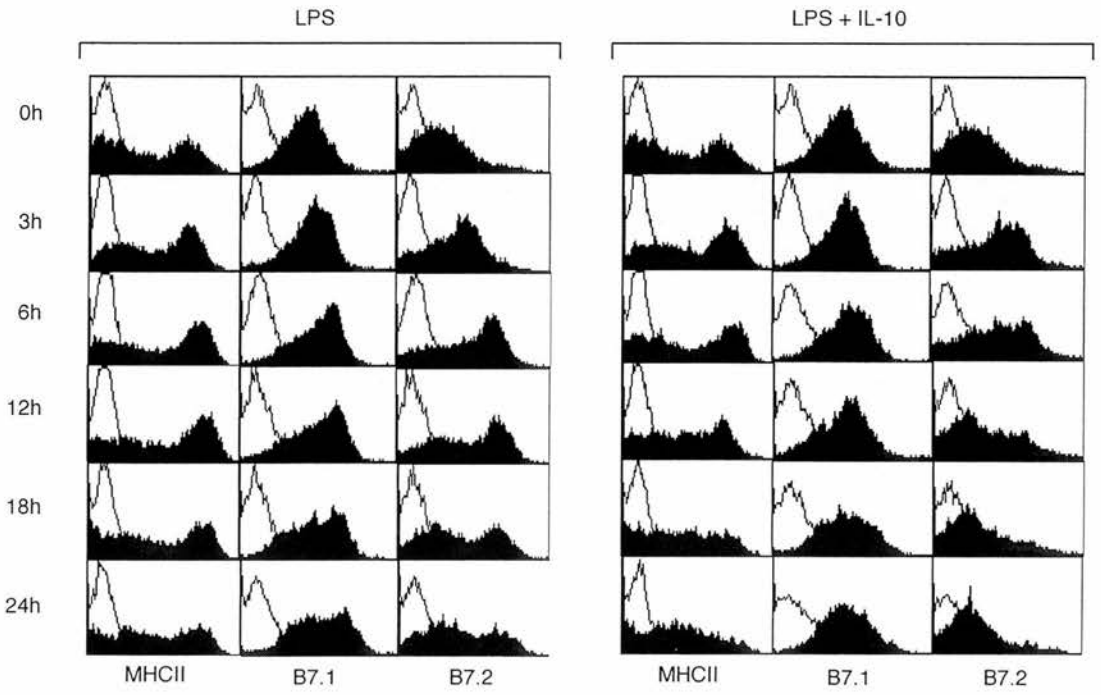

Figure 3.7 IL-10 accelerates DC activation kinetics. DC were stimulated with LPS or LPS plus IL-10 and samples removed from the culture at various times afterwards for analysis by flow cytometry. Open peaks indicate isotype controls. Data is representative of three independent experiments.

3.3.7 Post-activation death

The lifespan of DCs arriving in a lymph node is short; the final consequence of activation is death [155]. The rapid kinetics of IL-10 treated DCs could either speed them into an early death, or result in an extended post-activation 'exhaustion' with the cells then dying at the same time as those stimulated by LPS alone. To distinguish the two possibilities, the percentage of dead or dying cells was measured at various times after activation (fig 3.8). Unstimulated cells lived longer than either population given LPS; IL-10 treated DCs again behaved as mature cells. At early timepoints, however, their death rate was higher than that of cells in LPS alone, suggesting that IL-10 accelerates DCs through activation and on into death.

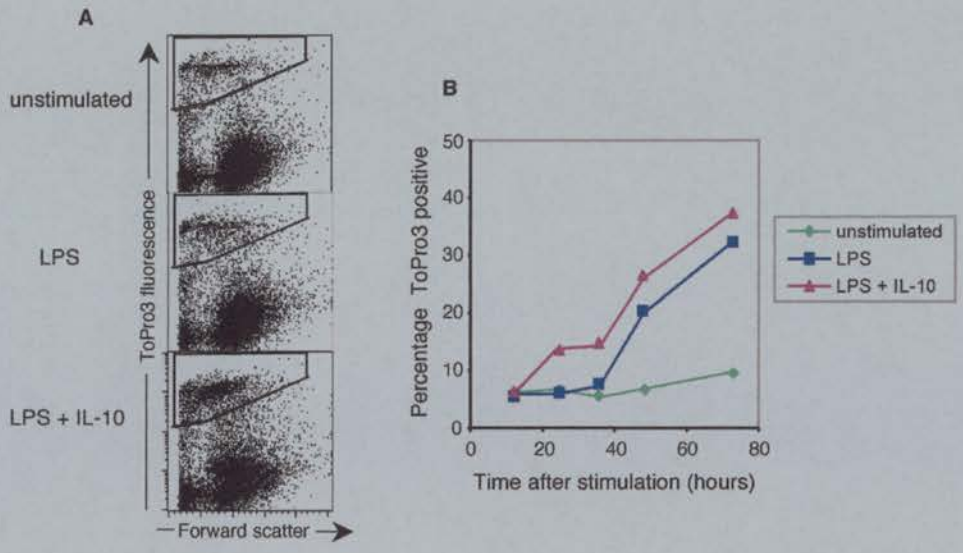

Figure 3.8 Cell death following activation. DCs were stimulated with or without LPS and IL-10 and stained with the membrane impermeant dye ToPro3 at various times afterwards. (A) An example of staining, taken after 24 hours' stimulation. The boxed region was used to define dead cells. (B) Percentage cell death as a function of time. Data is representative of four separate experiments.

3.3.8 Delayed addition of IL-10

Several groups have reported that DCs become resistant to the effects of IL-10 as they mature [205, 346, 351]. This is consistent with an inhibition of maturation, but it needed to be reconsidered in light of the kinetics of DC activation. DCs were stimulated with LPS and IL-10 was added either immediately or at various times afterwards. All cultures were analysed by flow cytometry 24 hours after the initial LPS (fig 3.9). As seen earlier, the LPS signal alone induced a relatively high level of B7.2 expression. When the LPS was given together with IL-10, that expression was downregulated. As the time delay was increased between the LPS stimulus and the addition of IL-10, the extent of suppression diminished.

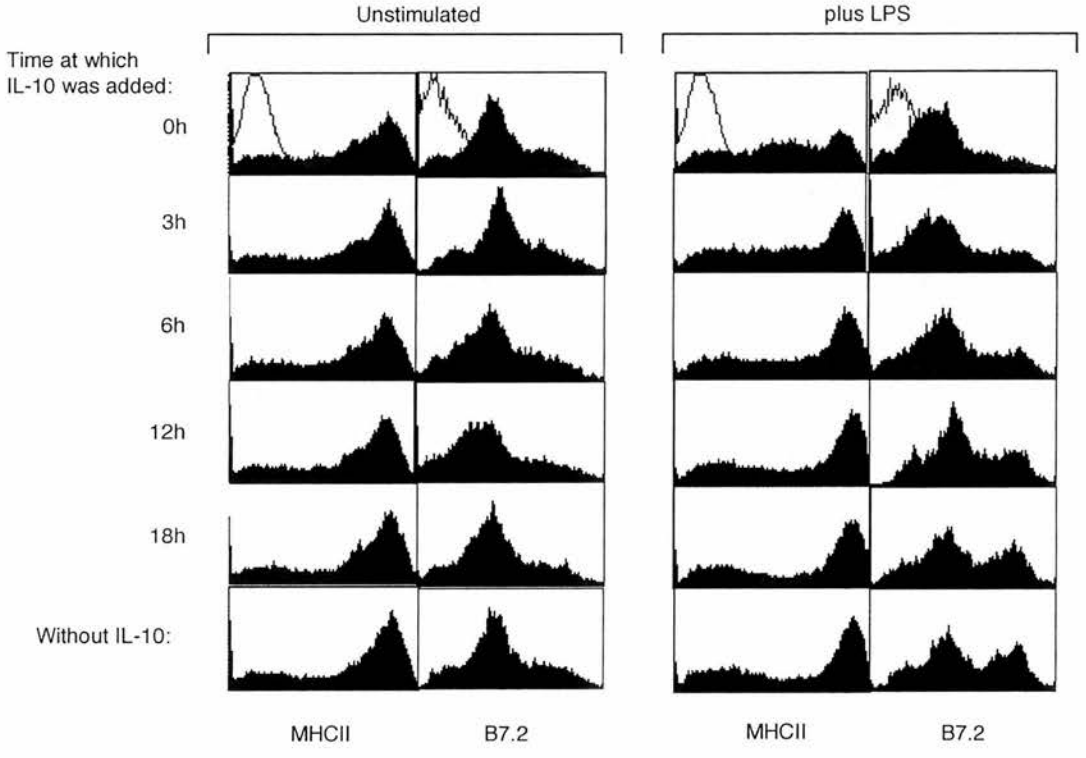

Figure 3.9 IL-10 can downregulate LPS-activated DCs. DCs were stimulated with LPS, or not, at 0h. IL-10 was added to both types of culture either immediately or at various times afterwards. Cells were harvested at 24h and their expression of surface markers measured by flow cytometry. Open peaks indicate isotype controls. Data is representative of two independent experiments.

This result is perhaps unsurprising given the experimental design: IL-10 added 18 hours after the LPS had considerably less time in which to act before the cells were taken for staining, compared to IL-10 given at the start of the timecourse. It does suggest, however, that shortly after stimulation DCs remain at least partially responsive to IL-10 mediated suppression. This window of opportunity appears to include the 6-8h timepoint at which LPS stimulated DCs are at the peak of their activation.

3.3.9 Cytokine deficiencies

To see whether IL-10 affected the quality of activation as well as its kinetics, DCs were examined first for their production of IL-12. IL-12 is a key dendritic cell cytokine, often used as an indicator of activation [352] [353] and proposed by Kalinski *et al.* to be the determinant of Th1 vs Th2 polarisation [10]. As seen by RT-PCR, both IL-12 *p40* and *p35* genes were strongly induced by LPS stimulation, with or without IL-10 (fig 3.10A). The response was short-lived, with downregulation of both genes apparent by 14h post-stimulation. Although these kinetics were affected by IL-10, with expression peaking earlier in its presence, the dominant effect was one of scale. This was reinforced when the samples were quantified by real-time RT-PCR (fig 3.10B). DCs activated in the presence of IL-10 did show a brief, early upregulation of IL-12 *p40*. Expression was maximal 2h after stimulation, 2000-fold above the pre-stimulation background, and had returned to baseline by 6h. This response was dwarfed by that seen with LPS alone, however; peak expression then was 10-fold higher. The *p35* subunit showed a similar pattern: although transcription was induced in the presence of IL-10, the effect was stronger in its absence.

When intracellular cytokine staining was used to visualise protein production during the first 6 hours post stimulation, the same IL-12 deficiency was apparent on two levels (fig 3.10C). The number of DCs making any IL-12 was lower in IL-10 treated cells than in those given LPS alone, and the IL-10 DCs also made less IL-12 on a per cell basis. Together this reinforces the difference in scale seen in the gene expression

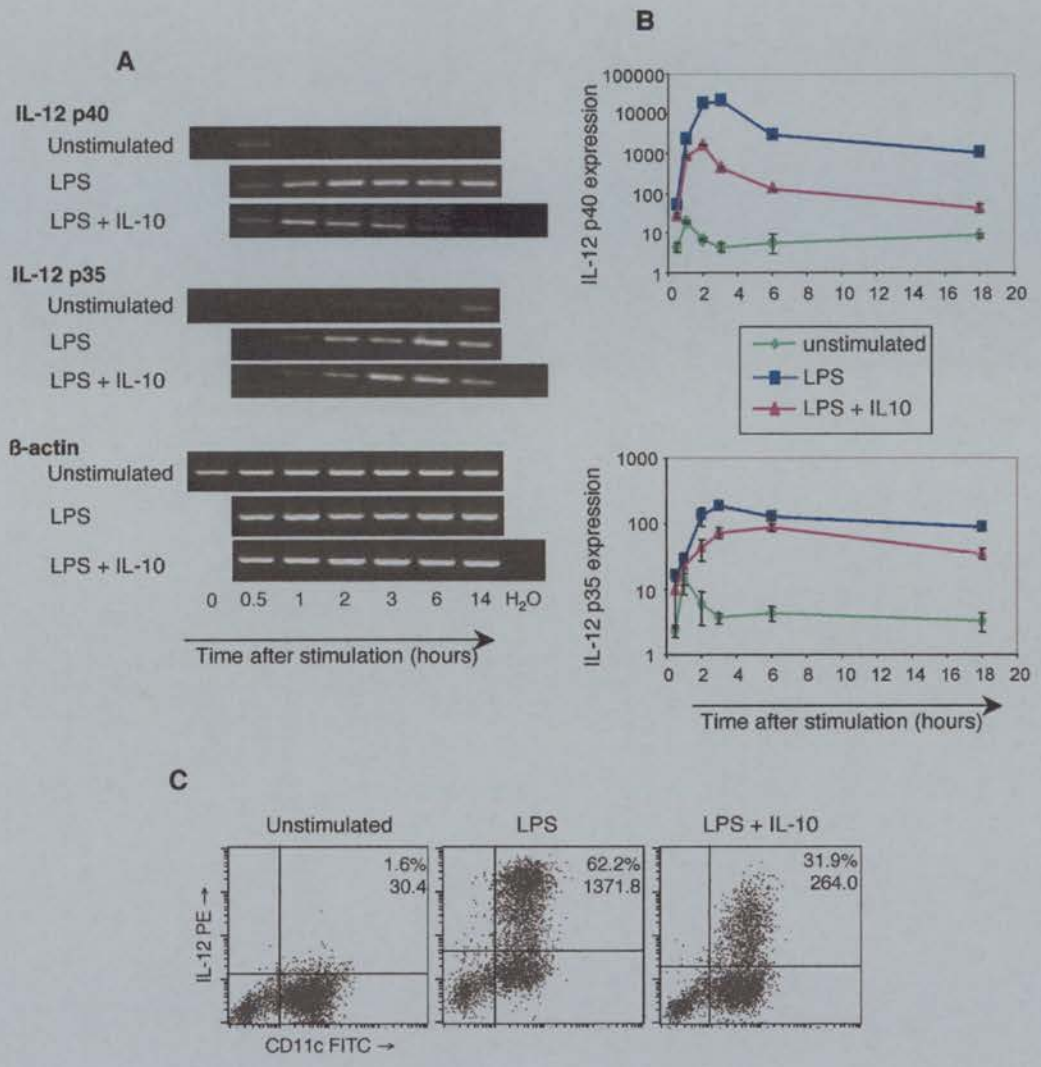

Figure 3.10 IL-12 expression. (A&B) DCs were stimulated with LPS and IL-10 and samples taken for RNA extraction at various times afterwards. (A) Expression of the two IL-12 subunits assessed by RT-PCR, using ß-actin for comparison. (B) Quantitative real-time RT-PCR. Y-axis scale indicates an *n*-fold increase above gene expression at 0h. Data is represented as the mean of triplicate reactions ± SEM. (C) Protein production was measured 6h after stimulation using intracellular cytokine staining. Numbers give the percentage of live CD11c+ dendritic cells positive for IL-12, ie. falling in the top right quadrant, and the y-axis mean fluorescent intensity of these cells. Data is representative of three independent experiments.

assays. One caveat to the protein data is that the staining antibody recognises the p40 subunit of IL-12 but cannot distinguish between monomers, bioactive heterodimers or potentially inhibitory homodimers.

That said, the main observation here appears to be that DC stimulated in the presence of IL-10, even when phenotypically activated, still make little IL-12. Reis e Sousa and colleagues have described an IL-12:IL-10 axis in which DC stimulated to produce one cytokine make little of the other [223]. McGuirk [112] and others [216, 354] have reported that IL-10 producing DCs induce a regulatory or anergic T cell response. I was interested therefore to see if the lack of IL-12 in my cells corresponded with an increase in IL-10. Figure 3.11 suggests not, neither at the level of gene expression nor of protein production. DCs activated by LPS alone express some IL-10 message but translate little protein; DCs stimulated in the presence of IL-10 express and translate virtually none.

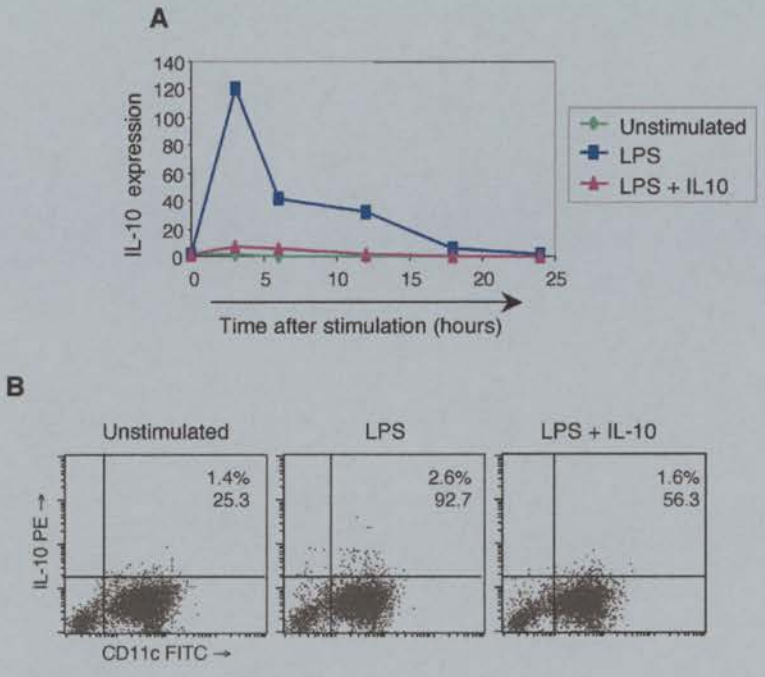

Figure 3.11 IL-10 production. DCs were stimulated with LPS and IL-10 and samples taken for RNA extraction or cytokine staining, as in figure 8. (A) IL-10 expression, assessed by real-time RT-PCR. Y-axis scale indicates an *n*-fold increase above gene expression at 0h. Data is shown as the mean of duplicate reactions. (B) Protein production, measured 6h after stimulation. Numbers give the percentage of CD11c+ cells positive for IL-10, and the mean fluorescent intensity of these cells. Data is representative of three separate experiments.

3.4 Discussion

Although it is clear that distinct DC subsets have distinct functions, this probably reflects a combination of lineage [189, 355, 356], stimulus [348], activation state [118] and environment [10, 12]. In a paper detailing five discrete DC populations separated according to degrees of expression of CD8α and CD11b, Pulendran *et al.* emphasised the complexity of the system [188]. He wrote,

> 'The data presented here highlight the need to view DC development ... as an interplay of distinct ontologic lineages with a maturational dependence on specific microenvironmental signals.'

By demonstrating different phenotypes in response to different cytokine signals, this chapter supports the argument for DC flexibility. The bone marrow derived DCs used were fairly heterogeneous, however, as evident in the range of MHCII expression on unstimulated cells (see fig 3.3, for example). Several alternative culture methods have been suggested in order to generate a more homogeneous, reliably immature source of DCs [357, 358], but in all of them, factors such as the gentleness of handling and the batch of culture medium can cause considerable variation. While my DCs were negative for CD8α and CD4 and, by virtue of their growth in GM-CSF, were most likely myeloid cells, it is a formal possibility that the different phenotypes arise not because of plasticity at the level of single cells, but in response to selective outgrowth of distinct precursors. In defence, there is very little proliferation of DC after day 7 of culture [335], which covers the lifespan studied here. Kapsenberg *et al.* tried to address this issue by skewing a DC population towards one extreme phenotype and then trying to reverse that function in the same cells. They found that, though initially open to polarisation, the DC became fixed and resistant to any opposing function. Their conclusion, however, was simply that DCs are one-shot cells with a limited lifespan (Kapsenberg, 1999, Metchnikoff lecture, Edinburgh, UK). The question remains open.

PGE_2 was described as a DC2 generating factor in Kalinski's seminal proposal of DC polarisation [10]. It is an inflammatory mediator associated with the enhancement of

humoral immunity [359, 360]. Its action results at least in part from its potent suppression of IL-12 production by APC [361, 362], which may [363] or may not [362] be due to induction of endogenous IL-10. In contrast to IL-10, however, PGE_2 has been reported to synergise with other inflammatory mediators in enhancing DC activation, specifically by augmenting upregulation of MHC and costimulatory molecules in response to IL-1β and TNFα. PGE_2 is also thought to be an important component of the ill-defined monocyte conditioned medium (MCM) used by Bhardwaj and colleagues as a positive control in the induction of DC maturation [83]. By looking only for PGE_2-mediated changes in the expression of DC markers or in the consequent proliferation of T cells, I perhaps missed the real impact of this reagent. Cytokine analysis would have been more revealing.

The same may be true of my assessment of IL-4. I observed slightly elevated expression of MHCII and B7.2 and T cell stimulation and saw hints of increased IL-12 by PCR. Since then, three reports have described IL-4 mediated promotion of IL-12 release by DCs, leading to a Th1 bias in the T cell response [364-366]. Although initially surprising, this may have evolved as a regulatory loop to prevent extreme Th2 reactions. A similar effect has been attributed to IL-13 [366]. There is supporting data *in vivo* too. IL-4 knockout mice are deficient in Th1 as well as Th2 responses [367]. Recombinant IL-4, rather than treating Th1 type autoimmunity, instead exacerbates symptoms in both experimental arthritis and uveoretinitis [368, 369]. In human atopic dermatitis, the T cell activity in the skin that is initially characterised by Th2 type cytokines later becomes dominated by IL-12 and IFNγ [370].

In contrast to IL-4, IL-10 is one of a number of factors reported to modulate DC function by trapping the DC in an immature state [205, 206, 346, 349, 351, 371]. Data presented here have shown that DC activated in the presence of IL-10 do respond to the LPS stimulus, downregulating antigen uptake and increasing expression of MHC and B7, but this activation is transient. The immunosuppressive action of IL-10 appears then to accompany some degree of DC maturation. The discrepancy may be explained by the different timescales examined. Beulens, Steinbrink, Kalinski and Sato all reported the apparently immature phenotype of IL-

10 treated DCs 48h or more after addition of IL-10 [205, 206, 346, 371]. While the kinetics of DC activation in human monocyte-derived DCs may be slower than in mouse bone marrow equivalents [117], this is still in full agreement with the observations presented here. That expression of activation markers is low at a particular time after stimulation does not necessarily mean that it has been low throughout the intervening period.

This is an idea that has been hinted at previously. Beulens *et al.* recorded uninhibited expression of the human DC maturation marker, CD83, in the presence of IL-10 [371]. De Smedt *et al.* noted that in splenic DC, IL-10 treatment did not prevent the upregulation of surface B7 or CD40 but did skew towards a Th2 outcome *in vivo* [372]. Rea *et al.* demonstrated that suppression of DC function by the steroid dexamethasone was concomitant with DC maturation [373]. The association between DC immaturity and tolerance, "signal 1 without signal 2" [374], was further challenged recently. Albert *et al.* reported that both cross-priming and cross-tolerance require mature DCs [375]. With both TCR engagement and costimulation in place, the T cell response was determined by an additional "signal 3". Like the data presented here, this suggests that DC maturation state alone does not dictate tolerance or immunity. In support, TNFα-matured DC have been shown to express high levels of MHC and costimulation and yet to elicit tolerance *in vivo* [111]. Maturation driven by a *B. pertussis* antigen gives a similar DC phenotype, and these cells guide the development of Tr1 regulatory T cells [112]. DC activated in the presence of IL-10 may share the same phenotype.

The nature of signal 3 remains uncertain. It was originally defined as a cytokine signal, directing the Th1/Th2 balance of the T cell response [10]. DC derived cytokines may contribute to T cell activation as well as polarisation [376], with IL-2 an intruiging new example [109, 245]. Both Menges [111] and McGuirk [112] used DC that were phenotypically activated but secreted little cytokine. The IL-10 treated DCs used here were also deficient in both IL-12 and IL-10. Lutz and Schuler [113] term such DC "semi-mature", a description that befits their poor performance in both cytokine release and T cell stimulation. The data presented in this chapter add a kinetic perspective: the high levels of MHC and B7 on our IL-10 treated DC are not

maintained. Their decline is rapid, with downregulation almost complete within 24h in the presence of IL-10. The surface of mature DC is considered relatively stable: the half-life of MHCII has been measured at over 100 hours [92]. This implies that the reduction in marker expression is an active process, not a passive loss. If the interaction between DC and responding T cells is normally a lengthy conversation [377], then the premature downregulation of key DC molecules may be sufficient to limit T cell proliferation. Their activation would literally be aborted [378].

If DC regulation was purely kinetic, the T cell outcome would be dictated by the stage of differentiation that the DC had reached as it arrived in the lymph node. If it met a specific T cell at the height of activation, then strong immunity would result; if it was past its peak, the consequent immune response would be different, perhaps even tolerant. This is an extension of Lanzavecchia *et al.*'s description of DC exhaustion [118, 379]. Simplistically, it predicts that the same activation should occur in the presence and absence of IL-10 with the only difference being one of timing. This is indeed what happens in terms of surface phenotype, but analysis of the IL-12 expression of the two DC populations revealed qualitative differences. Temporal regulation and alternative activation may be complementary. The kinetic profiles of the individual costimulatory molecules differ: the variation in expression is more pronounced for B7.2 than B7.1, for example. The balance of costimulation signals provided by DC will consequently change over time, influencing the T cell outcome [262, 263]. A similar argument can be made for IL-12. Bioactive IL-12 is a 70kD heterodimer comprised of p40 and p35 chains, encoded by separate genes. In DCs, regulation appears to focus on the p35 subunit [365, 380] and an excess of p40 can form inhibitory homodimers [381]. Data in this chapter suggests that the induction of p35 expression in response to LPS is delayed relative to that of p40. The changing ratio of the two subunits will determine the amount of functional IL-12 produced over time.

How long does it take for a DC to respond to pathogenic signals in the periphery and to arrive in the lymph node? Norbury *et al.* observed antigen-laden DCs in the draining lymph node within 6h of injecting virus into the footpad [382]. Both DC number and antigen presentation then declined rapidly, suggesting a relatively short

timescale. Experiments using an adoptive transfer of DO11.10 T cells reported maximal production of IL-2 by lymph node T cells 6h after intravenous administration of peptide [383], and by 12h following subcutaneous injection of protein antigen [384]. The low levels of MHCII and B7 on the surface of DC 24h after stimulation in the presence of IL-10 appear less relevant than the high levels that we see at 6h. The establishment of tolerance by IL-10 treated DC [205, 351] may not be a default response caused by the immaturity of the DC, but a reaction to definite signals from fully differentiated APC.

In summary, IL-10 has a powerful influence on DC function. The data presented in this chapter suggest that this is achieved not by preventing DC maturation, but by controlling the kinetics and cytokine profile of their activation. I next wanted to assess the impact this might have on the responding T cells.

Chapter 4 - The T cell response to IL-10 DCs *in vitro*

4.1 Introduction

Adaptive immunity is distinguished by its vast repertoire of antigen specificities. This creates a formidable defence against pathogens, but it comes at a cost: the random generation of antigen receptors allows the development of self-reactive lymphocytes, capable of attacking the body's own tissues with the same destructive efficiency normally used to purge infections. Tolerance mechanisms have evolved alongside immunity to protect us from such 'horror autotoxicus' [220, 385]. Central tolerance prevents the development of autoreactive cells by deleting those that respond to self peptides displayed in the thymus [386, 387]. The system is leaky, however, and cells with low affinity receptors or whose specific antigen is absent from the thymus can survive and escape into the periphery [82, 275]. Peripheral tolerance exists to prevent the activation of these survivors. It comes in several guises. Deletion [388, 389], anergy [290], suppression [390] and deviation [298] have all been described.

The inhibition of T cell responses is currently a topic of intense interest. Several populations of regulatory T cells have recently been described, including CD25+ Treg cells, IL-10 dependent Tr1 cells and TGFβ secreting Th3s [305, 317, 323]. While their surface markers and their means of suppression appear to be different, all three groups are characterised by a limited proliferation in response to antigen. This is reminiscent of T cell anergy, defined as a long-term state of hyporesponsiveness [292] and also proposed as a mechanism of peripheral tolerance [289]. Anergy was originally understood to be a result of signal one without signal two, TCR triggering without adequate costimulation [391]. Immature DCs express sufficient MHC to engage TCRs [217], but with very little B7.1, B7.2 or CD40 [358]. Thus, while the activation of dendritic cells initiates immunity, the steady-state migration of immature DCs is thought to maintain peripheral tolerance [328].

The link between IL-10 treated DCs and T cell tolerance, then, appears to be a simple one: IL-10 inhibits the maturation of DCs, preventing their upregulation of costimulatory molecules and rendering responsive T cells anergic [205]. The data presented in chapter 1 suggest that this may not be the whole story. Six hours after activation, DCs stimulated in the presence and absence of IL-10 both express high levels of MHCII, B7.1 and B7.2. This chapter considers the impact of these DCs on an *in vitro* population of responding T cells. Experiments show that even early after stimulation, with high levels of MHC and B7, IL-10 treated DCs fail to prime T cells as effectively as their LPS-matured counterparts. This initial interaction with an IL-10 treated DC appears to limit both the proliferation and the cytokine production of the T cells upon subsequent rechallenge. Although both DC populations are phenotypically activated, they direct quite different T cell responses.

4.2 Approach

Immature DCs were harvested on day 7 of culture and stimulated for 6 or 24h with LPS or LPS and IL-10. They were washed, pulsed with ova peptide and mixed with CD4+ cells from DO11.10 TCR Tg mice. The resulting T cell proliferation and activation marker expression in these primary co-cultures was analysed by ^{3}H-thymidine incorporation and flow cytometry. In addition, some T cells were kept in culture for 7d, in the presence of exogenous IL-2, before being recovered and restimulated. These secondary cultures were assessed by recording their proliferation in response to peptide presented on irradiated APCs, and by analysing their cytokine expression after stimulation with PMA and ionomycin.

4.3 Results

4.3.1 T cell stimulation at 24h

As described in chapter 3, DCs activated with LPS and IL-10 retained only very low levels of MHC and B7 on their surface 24h later (see fig 3.3). Correspondingly, they elicited only limited T cell proliferation (figs 3.3 and 4.1). Like their surface marker expression, their T cell stimulation was below that of the unstimulated controls, and although the range of peptide concentrations did not reach saturation, this deficiency was not overcome by increasing the antigen dose (fig 4.1). In contrast, DCs activated with LPS alone induced high levels of T cell proliferation (fig 4.1).

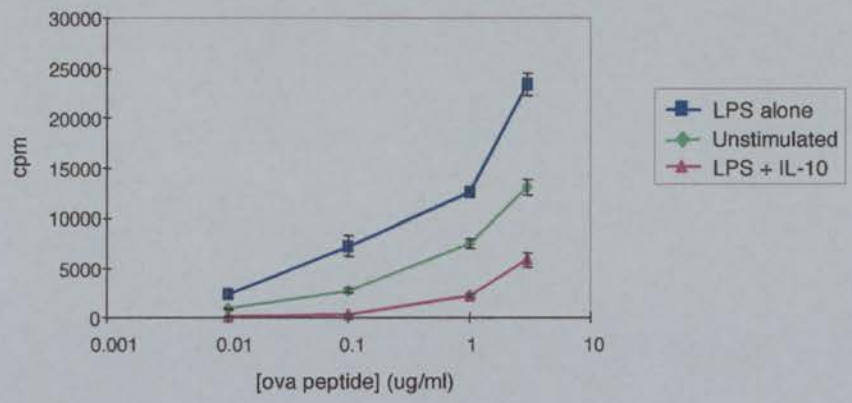

Figure 4.1 IL-10 treated DCs fail to stimulate T cell proliferation. DCs were harvested at day 7 of culture and replated in medium alone (unstimulated), with LPS or with LPS and IL-10. After 24h cells were pulsed with ova peptide and mixed with CD4+ cells from DO11.10 TCR Tg mice. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM and is representative of three independent experiments.

4.3.2 T cell stimulation at 6h

The lack of proliferation seen in response to DCs activated in the presence of IL-10 for 24h or more has been widely reported [205, 346, 351, 392] and is easily explained in terms of their limited expression of MHC and costimulatory molecules. At 6h post-activation, however, the surface phenotype of these DCs appears largely equivalent to that of DCs matured with LPS alone (see fig 3.5). Surprisingly then, even when the DCs were mixed with T cells 6h after activation, the IL-10 treated population still displayed a marked deficiency in T cell stimulation. The responding T cells proliferated no more than they did in response to unstimulated DCs, whereas LPS-activated DCs induced much stronger T cell proliferation (fig 4.2).

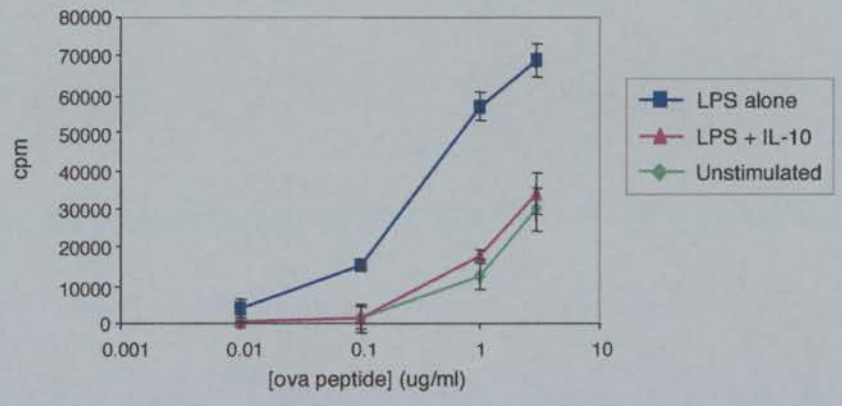

Figure 4.2 Even at 6h, IL-10 treated DCs stimulate a limited T cell response. The different DC populations were assessed in a T cell proliferation assay as in figure 4.1, but here the DCs were stimulated with LPS ± IL-10 for only 6h before being pulsed and mixed with T cells. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM and is representative of four independent experiments.

4.3.3 Addition of exogenous IL-12

Since IL-10 treated DCs stimulated only limited T cell proliferation at 6h post activation, despite high levels of MHCII, B7.1 and B7.2, some other aspect of the

DC phenotype had to be responsible. IL-12 was an obvious candidate: it is a key component of DC activation and it was expressed at clearly different levels by DCs stimulated in the presence or absence of IL-10 (see fig 3.10). IL-12 was originally described as a T cell growth factor [393, 394] and it has also been shown to support B cell proliferation [395].

Figure 4.3 shows, however, that IL-12 is not the explanation for the lack of T cell proliferation seen here. The addition of exogenous IL-12 to the cytokine deficient, IL-10 treated DCs did not enhance their T cell stimulation, and the addition of blocking antibodies against IL-12 to the cytokine sufficient, LPS-activated DCs did not inhibit theirs (fig 4.3).

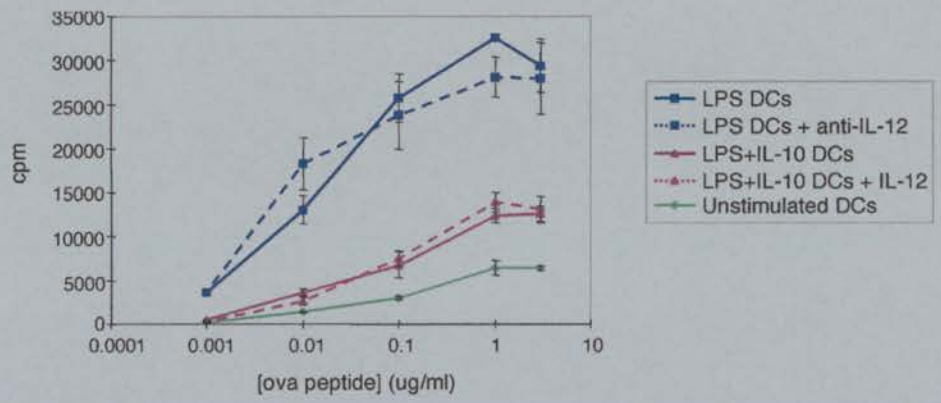

Figure 4.3 IL-12 does not affect T cell proliferation. DCs were harvested at day 7 of culture and replated in medium alone, with LPS or with LPS and IL-10. After 6h DCs were pulsed with ova peptide and mixed with DO11.10 CD4+ cells. Some co-cultures were supplemented with either recombinant IL-12 or with an anti-IL-12 blocking antibody, as indicated. Data is shown as the mean of triplicate wells ± SEM.

4.3.4 T cell activation markers

The low level of T cell proliferation in response to IL-10 treated DCs could indicate either inefficient priming, leaving a higher percentage of T cells ignorant and naïve, or an active suppression of the T cell response. To distinguish the two possibilities, the T cells were assessed first in terms of their expression of activation markers.

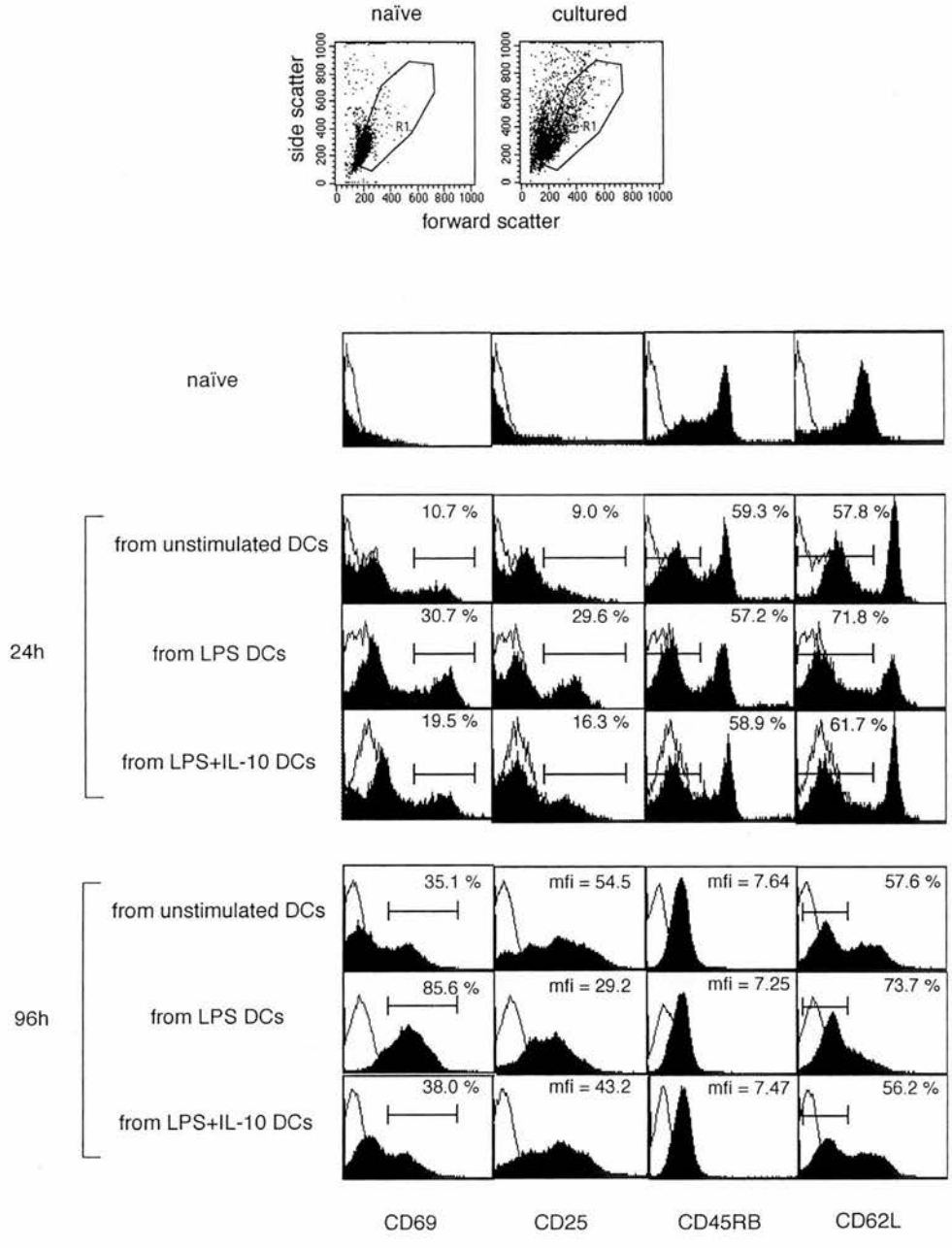

Figure 4.4 T cell activation markers. DCs were harvested at day 7 of culture and replated in medium alone, with LPS or with LPS and IL-10. After 6h DCs were pulsed with ova peptide and mixed with DO11.10 CD4+ T cells. Samples were removed from the co-cultures for analysis by flow cytometry either 24 or 96h later, as indicated. "Naïve" cells were CD4+ T cells purified from a DO11.10 mouse and stained immediately. All histogram plots are gated on R1, which was set on forward and side scatter characteristics to include all live lymphocytes, both resting and blasting. It is shown at the top of the figure. Percentages give the proportion of gated cells falling within the marker shown, and mfi refers to the mean fluorescent intensity of all gated cells. Data is representative of three separate experiments.

Naïve T cells were mixed with antigen-pulsed DCs that had been stimulated for 6h, and samples then taken for analysis by flow cytometry. After 24h of co-culture, all three groups of DCs had induced markers of T cell activation: CD69 and CD25 were upregulated and CD45RB and CD62L downregulated, in comparison to freshly isolated naïve CD4+ cells (fig 4.4). The degree of activation fitted well with the proliferation data shown earlier (fig 4.2). The T cell population stimulated by LPS-matured DCs contained more $CD69^{hi}$, $CD25^{hi}$ and $CD62L^{lo}$ cells than the populations stimulated by DCs given LPS and IL-10 or DCs left in medium alone (fig 4.4). The latter two groups appeared similar in phenotype, as they did in proliferation, despite the difference in expression of MHC and B7 on the dendritic cells.

To assess the phenotype of the T cells later in their response, samples were also taken after 96 hours of co-culture. Expression of CD69 and CD62L mirrored that seen at 24h, with a greater percentage of activated T cells in the group stimulated by LPS DCs than in either of the others (fig 4.4). The CD25 staining was particularly interesting. Transient display of CD25, the α chain of the IL-2 receptor, is commonly used as a T cell activation marker [396], but sustained expression has been associated with a population of regulatory T cells shown *in vitro* and *in vivo* to suppress other T cell responses [305, 397]. While almost all cells in fig 4.4 expressed some CD25 at 96h, the intensity of staining was higher in the unstimulated and LPS+IL-10 groups than in the LPS alone set. Although intruiging, this trend was not consistent between experiments.

4.3.5 T cell restimulation

To distinguish further between a failure to prime and an active suppression, T cells responding to IL-10 treated DCs were maintained in culture in the presence of exogenous IL-2, before being recovered and rechallenged with peptide presented on irradiated APC. The reasoning was that if the T cells had been left ignorant by inefficient priming, then the competent APC should elicit normal, primary-type proliferation, whereas if the T cells had been tolerised, then their response should be inhibited. The result is given in figure 4.5. While T cells recovered from culture

with unstimulated DCs showed surprisingly strong proliferation, above even that of the LPS-activated DCs, T cells originally stimulated by IL-10 treated DCs were much less responsive to restimulation.

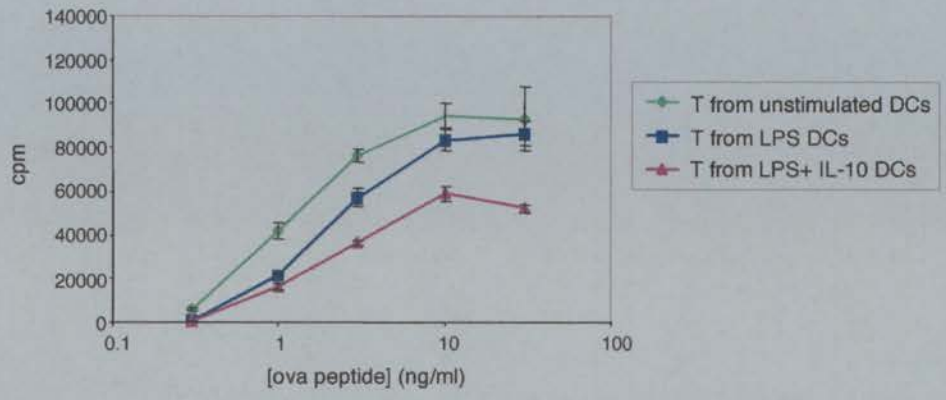

Figure 4.5 Recovered T cells respond differently to restimulation. DCs were harvested at day 7 of culture and replated in medium alone, with LPS or with LPS and IL-10. After 6h DCs were pulsed with ova peptide and mixed with DO11.10 CD4+ cells. 3d later, the cells were spun through lympholyte and replated in fresh medium containing IL-2 (see section 2.7.2). 7d after the original co-culture, cells were collected, counted and mixed with irradiated splenocytes. Ova peptide was added and proliferation measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM and is representative of three independent experiments. The corresponding primary response curve is shown in fig 4.2.

4.3.6 Cytokine profiles

To see whether the initial interaction with IL-10 treated DCs affected the cytokine secretion of the responding T cells as well as their proliferation, T cells were recovered and restimulated with PMA and ionomycin in the presence of a Golgi block. Their cytokine patterns were then assessed by intracellular staining. T cells stimulated by LPS DCs predominantly produced IFNγ and expressed little IL-4 or IL-10 (fig 4.6), in keeping with the IL-12 made by these DCs (see fig 3.10). T cells co-cultured with unstimulated DCs, which expressed very little IL-12, exhibited a Th2 profile, with a marked reduction in IFNγ and increased levels of both IL-4 and IL-10. In contrast, while the IL-12 secretion of DCs stimulated with LPS and IL-10

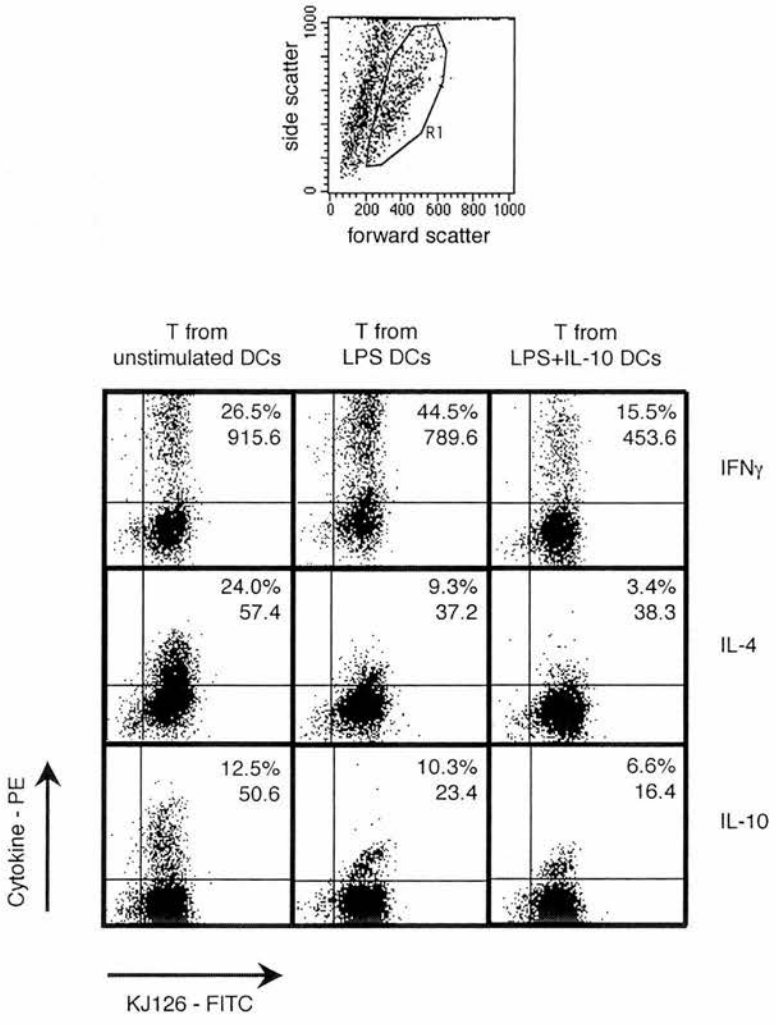

Figure 4.6 T cell cytokine profiles. DCs were harvested at day 7 of culture and replated in medium alone, with LPS or with LPS and IL-10. After 6h DCs were pulsed with ova peptide and mixed with DO11.10 CD4+ cells. 3d later, the cells were spun through lympholyte and replated in fresh medium containing IL-2 (see section 2.7.2). 7d after the original co-culture, cells were stimulated with PMA and ionomycin and their cytokine expression measured by intracellular staining and flow cytometry. All dot plots are gated on R1, set by forward and side scatter to include blasting lymphocytes and shown at the top of the figure. Numbers give the percentage of gated events falling in the top right quadrant and the y-axis mean fluorescent intensity of these cells. Data is representative of three separate experiments.

was intermediate between that of the unstimulated and LPS-matured groups, the responding T cells were characterised by a general cytokine deficiency. Their expression of IFNγ, IL-4 and IL-10 was well below that of either of the other groups.

4.4 Discussion

Even early after activation, despite their similarly high expression of surface MHC and B7, DCs activated in the presence and absence of IL-10 are not equivalent. This was demonstrated in relation to the DC in chapter 3 and is illustrated here by its influence on the T cell response. IL-10 treated DCs stimulate only limited T cell proliferation and the responding T cells display low levels of activation markers, are less sensitive to restimulation and make little cytokine.

There are several possible interpretations of this data. Looking at early expression of T cell activation markers suggested that both unstimulated and IL-10 treated DCs prime inefficiently, leaving a significant number of unactivated, naive T cells. Unstimulated or immature DCs are not prepared for T cell interaction. The classical Langerhans Cell paradigm has immature DCs in the periphery physically separated from naïve T cells in the lymph node [88, 89], and some form of stimulation is required to trigger the DC to change its chemokine receptors and migrate [398]. CCR7 expression, which controls entry to the T cell zones of secondary lymphoid tissue, is only upregulated on mature or maturing DCs [398, 399]. In addition, the adhesion molecules, TCR ligands and costimulators that a T cell must see on an APC in order to connect, engage and signal are all expressed at low levels on immature DCs [7]. Maturation is associated with a ten-fold escalation of transport of new MHCII molecules to the plasma membrane [104] and a further ten-fold increase in the half-life of MHCII once there [92]. The opportunity for conversation between an immature DC and a T cell may only be fleeting.

In contrast, IL-10 treated DCs express high levels of MHC and B7 and yet still fail to stimulate strong T cell proliferation. Why? What is the purpose of upregulating these molecules if not to engage T cells? One explanation may lie in the design of

the experiment. The 6h timepoint was identified as the peak of B7.2 expression in IL-10 treated DCs by giving LPS and IL-10 simultaneously. If signalling through the IL-10 receptor happens more slowly than the rapid LPS response [400] then the first message received by the nucleus will be a direction to upregulate activation markers, and the downmodulatory influence of IL-10 will only come later. The early burst of MHC and B7 would not then represent a decisive activation state but more an inevitable consequence of cell physiology.

A more appealing explanation is that the presence of MHC and B7 enables a productive interaction with the T cell, but one that delivers a non-proliferative signal. Several examples of negative signalling have been described. The best characterised is the interaction between B7.1 and B7.2 on the APC and CTLA4 on the T cells [401]. CTLA4 binds the B7 ligands with greater affinity than CD28 [402, 403], but CD28 is constitutively expressed while the appearance of CTLA4 requires T cell activation [404] [405]. The first contact is therefore between B7 and CD28, enhancing T cell activation, driving an initial burst of proliferation but also causing upregulation of CTLA4 and consequent termination of the T cell response [406, 407]. The mechanisms used by CTLA4 to suppress T cell activation are still being elucidated. As an alternative receptor for B7.1 and B7.2, it competes with CD28 for the same ligands. Downstream signalling from CTLA4 uses the same phosphatidylinositol 3-kinase (PI-3K) and serine-threonine phosphatase molecules as required by CD28, so competition also occurs intracellularly [408]. CTLA4 signalling can directly inhibit TCR signal transduction, by activating an enzyme that removes phosphates from the ITAM motifs in the TCR complex [409]. Finally, CTLA4 inhibits T cell progression through the cell cycle, particularly elongating S phase [410]. Engagement of CTLA4 is a positive signalling event with a negative outcome on proliferation.

Recently new members of the B7 costimulator family have been described and some of these too have been attributed regulatory function. B7h (also known as B7RP-1 and B7H2) is widely expressed on DCs, other APC and non-haematopoietic cells [411, 412]. Its ligand, ICOS, is upregulated on T cells after activation and hence is thought to be important in directing effector function rather than during priming

[413]. Its role in T cell regulation is less clear. Blocking ICOS costimulation during the induction of autoimmunity exacerbates EAE [414, 415]. This may reflect the lack of Th2 polarisation thought to be encouraged by the B7h-ICOS interaction [416]. ICOS signalling has also been shown to contribute to Th1 induction [417], however, and while the EAE studies were performed *in vivo*, the *in vitro* data is contradictory. Priming myelin specific T cells *in vitro* in the absence of ICOS signalling reduced their ability to elicit EAE when transferred back into mice. This was associated with an inhibition of both IFNγ and IL-10 responses [418]. The discrepancy is puzzling. Two review articles have suggested that it indicates a role for ICOS costimulation in the induction of regulatory T cells *in vivo* [419, 420].

Perhaps more exciting are the molecules PD-L1 (also known as B7H1) and PD-L2 (also called B7DC), both expressed on DC and both shown to interact with PD-1 on the T cell [421, 422]. Stöckl *et al.* recently described the use of anti-PDL1 blocking antibody to manipulate T cell responses. While IL-10 treated DCs induced T cell anergy, coating these DCs with anti-PDL1 restored proliferation (Stöckl, 2002, ENII conference, Lez Embiez, France). This is strong evidence that the T cell response to IL-10 treated DCs is not simply due to a lack of costimulation. Screening these DCs for expression of B7 homologues and other inhibitory signals would be very interesting.

Analysis of DC cytokines other than IL-12 and IL-10 might also be revealing. TNFα, IL-6, IL-1β and IL-2 are all released from classically activated DCs and are therefore good candidates for a missing signal from IL-10 treated DCs [423, 424]. IL-2 is particularly significant because one of the defining characteristics of T cell anergy is the inability to transcribe IL-2 [425]. Addition of exogenous IL-2 has been shown to overcome anergy in vitro [426]. This has two important implications. It may explain why my demonstration of "hyporesponsiveness" was somewhat muted. The T cells from IL-10 DCs did show a reduced proliferation in response to rechallenge, and their cytokine expression was limited, but particularly in a transgenic system where almost all the T cells are of a single specificity, a whole population effect would have been more convincing. In both types of restimulation assay, however, exogenous IL-2 was added to the T cell cultures (see section 2.7.2).

This may have undermined any ability of the DC to induce anergy, and a comparison of cultures with and without IL-2 would have been useful. The importance of IL-2 also questions the relevance of this type of anergy to peripheral tolerance *in vivo*, however. If the IL-2 produced during any normal immune response could trip anergic, self-reactive T cells into action, then it is surprising that more of us do not suffer autoimmunity.

Functional inactivation has found favour once again with the current interest in regulatory cells [297, 302]. CD25+ regulatory cells are naturally selected in the thymus and form 5-10% of circulating peripheral T cells [427]. Thymectomy of 3 days old mice, before CD4+ CD25+ cells have emerged into the periphery, leads to widespread autoimmunity [428, 429]. Pathogens have also been reported to subvert DC function to generate inhibitory T cells [112]. This is not only a cunning survival strategy for the pathogen, it also has considerable potential for therapeutic exploitation. The induction of a regulatory T cells by IL-10 treated DCs is an attractive idea. The difference in proliferation between restimulated T cells from IL-10 treated and LPS-matured DCs could just be the difference between a primary and a secondary response, if the original lack of T cell stimulation by the IL-10 treated DCs was a failure to prime, but it could also represent the limited proliferation seen in anergic [391] and regulatory T cell populations [317]. The use of CFSE staining may have been enlightening here, since unlike ^{3}H-thymidine, CFSE can distinguish between few cells dividing normally and many cells dividing only once.

The limited rechallenge response of T cells stimulated by IL-10 treated DCs was also characterised by reduced cytokine production. The difference in the T cell reactions to unstimulated and IL-10 treated DCs here was interesting: while unstimulated DCs gave rise to a Th2 biased profile, IL-10 DCs elicited little T cell cytokine of either type. Do the IL-10 DCs make enough IL-12 to prevent the outbreak of a Th2 response but too little to drive a strong Th1 differentiation? Is that sufficient to make them suppressive? Figure 4.3 suggests not, since supplementing IL-10 treated DCs with exogenous IL-12 does not enhance their T cell stimulation, but T cell function may not be a direct correlate of proliferation. Importantly, the T cells recovered from cultures with IL-10 treated DCs did not secrete high levels of IL-10, despite the

association between this cytokine and inhibitory T cells [317]. Limited cytokine production is a feature of anergic T cells, however [289, 296].

The real need here is for some functional assessment of the recovered T cells. Testing for suppressive action by titrating them into another proliferation assay would reveal much about their phenotype. It may be that several rounds of restimulation *in vitro* are needed to generate a truly inhibitory population [317, 329].

This is the mixed blessing of *in vitro* data: the systems are simple, offering exact control of variables and the potential to tease apart complex mechanisms. As a consequence, though, the culture conditions used have a major influence on the results obtained. Although enlightening, their relevance to physiology can be debatable. To address this, it was decided to establish an adoptive transfer system to test the different DC phenotypes *in vivo*.

Chapter 5 - The T cell response to IL-10 DCs *in vivo*

5.1 Introduction

The ability to generate substantial numbers of dendritic cells from bone marrow or blood-borne precursors [335, 347, 358] made therapeutic application an exciting possibility [430, 431]. Early experiments exploited the potent immunogenicity of mature DCs to augment vaccination regimes [432] and boost anti-tumour responses [433, 434]. DCs are multifunctional cells, however, capable of directing a variety of T and B cell responses [10, 435]. *In vitro* manipulation before injection offers the potential to deliberately engineer the outcome of an immune response. Data presented in chapters 3 and 4 suggest that DCs stimulated in the presence of IL-10 rapidly acquire an active phenotype that fails to elicit strong T cell proliferation. This chapter begins to assess their therapeutic promise by asking first whether IL-10 treated DCs can limit T cell expansion *in vivo* as shown *in vitro*, and second whether this initial DC:T cell interaction has any lasting consequence for T cell activity.

The first experiment was theoretically simple: I wanted to activate DCs with LPS alone or with LPS and IL-10, pulse both populations with antigen and inject them into mice, allowing them to interact with T cells *in vivo*. The difficulty with this approach lay in detecting the response. The frequency of T cells of any given specificity is extremely low in a wildtype animal; Tse *et al.* estimated that T cells of a single specificity make up less than $^{1}/_{100\,000}$ of the circulating pool, rising to only $^{1}/_{10\,000}$ even after immunisation [436]. To analyse 1000 of these T cells would require a starting population of $\sim 1 \times 10^{7}$ cells for each data point, the equivalent of the draining lymph node cells from one immunised mouse [437]. One of the attractions of TCR transgenic mice, then, is that they have T cells of almost entirely a single specificity. In DO11.10 mice, approximately 75% of T cells express both chains of the transgenic receptor [334, 438]; in DO11.10 mice on a $Rag^{-/-}$ or a SCID background, preventing endogenous rearrangement of the TCR-Vα chain, the figure is almost 100% [439]. Immune responses are not made any easier to detect, however. Intact TCR transgenic mice respond very poorly to attempts to induce

either immunity or tolerance [195, 440]. The overwhelming frequency of responsive T cells appears to inhibit normal development of immune responses.

Marc Jenkins and colleagues devised a clever compromise. By transferring a limited cohort of DO11.10 T cells into a wildtype BALB/c recipient, they achieved a situation where ~0.5% of T cells in the lymph nodes carried the transgenic specificity [195, 437, 439]. This is still above a typical baseline for peptide-specific responses, but it is not dissimilar to the frequency of alloreactive T cells in a normal individual [441] and, crucially, it allows the development of both immunity and tolerance [195]. One of the key advantages of this adoptive transfer system is the availability of a monoclonal antibody, KJ126, which specifically recognises the transgenic TCR of DO11.10 mice [442]. Using KJ126, both flow cytometry and immunohistochemistry can be exploited to follow the progress of an immune response *in vivo* [195, 443, 444]. In the experiments presented here, DO11.10 transfers were used to examine the responses generated by DCs stimulated in the presence or absence of IL-10. The T cell expansion triggered by IL-10 treated DCs was shown to be smaller than that induced by DCs activated by LPS alone, mirroring the differences seen *in vitro* in chapter 3. This initial contact with IL-10 treated DCs *in vivo* also appears to render T cells hyporesponsive to subsequent antigenic challenge.

5.2 Approach

A DO11.10 adoptive transfer system was established, working largely from Jenkins's published protocols [195, 439] with DC immunisation procedures adapted from Pulendran *et al.* [166] and Maldonado- Lopez *et al.* [152]. To examine the performance of the different DC phenotypes, DCs were harvested after 7 days of culture and stimulated for 6h with LPS or LPS+IL-10. The cells were washed, pulsed with ova peptide, washed again and injected into mice that had been adoptively transferred 24h earlier with a cohort of DO11.10 T cells. The experimental outline is illustrated in fig 5.1. Mice were either killed at day 5 after

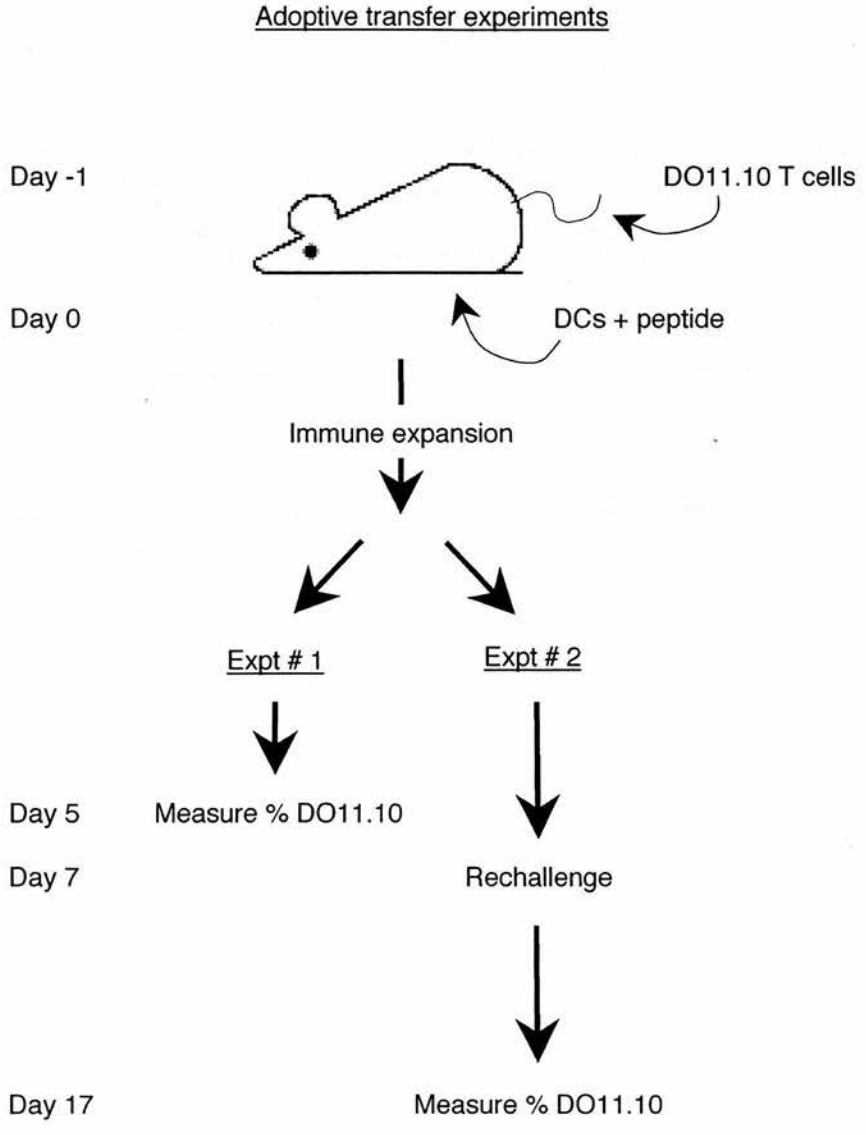

Figure 5.1 Experimental outline. An adoptive transfer system was established in which Balb/c mice received a cohort of DO11.10 TCR Tg T cells before being immunised with different DC populations, each coated with ova peptide. The mice were either sacrificed after five days or rechallenged *in vivo* and sacrificed at day 17. Their response to ova was measured by recording any expansion of the DO11.10 population and assessing its ability to respond to ova peptide *in vitro*.

priming to assess T cell expansion at the peak of the primary response, or left intact and instead rechallenged *in vivo* on day 7. This second immunisation was given as a duplicate shot of LPS-activated DCs or as a s.c. injection of peptide in CFA, and in both cases peak expansion of the secondary response was measured 3 days later, on day 10. Some mice were kept until day 17 and their spleen or lymph node cells then restimulated *in vitro*.

5.3 Results

5.3.1 DO11.10 T cells before and after transfer

To identify a convenient source of transgenic T cells, an initial comparison was made of adoptive transfers of either unseparated lymph node cells or CD4+ enriched splenocytes. The lymph nodes of DO11.10 mice were collected, pooled and immediately prepared for injection. Spleens from the same animals were lysed of red blood cells and depleted of CD8+ and MHCII+ contaminants by magnetic separation (see section 2.4.2). The purities of the two preps are illustrated in fig 5.2.

Both populations were transferred into wildtype BALB/c recipients by i.v. injection into the tail vein. Two concentrations of CD4+ splenocytes were used. The transferred mice were sacrificed 24h later and their spleens and lymph nodes (pooled popliteal, inguinal, brachial, axillary and cervical) examined by flow cytometry (figs 5.3 and 5.4). While the percentage of CD4+ KJ126+ cells in an untransferred BALB/c mouse was undetectable, each of the transfer recipients displayed a small but distinct double positive population. The transfer had worked.

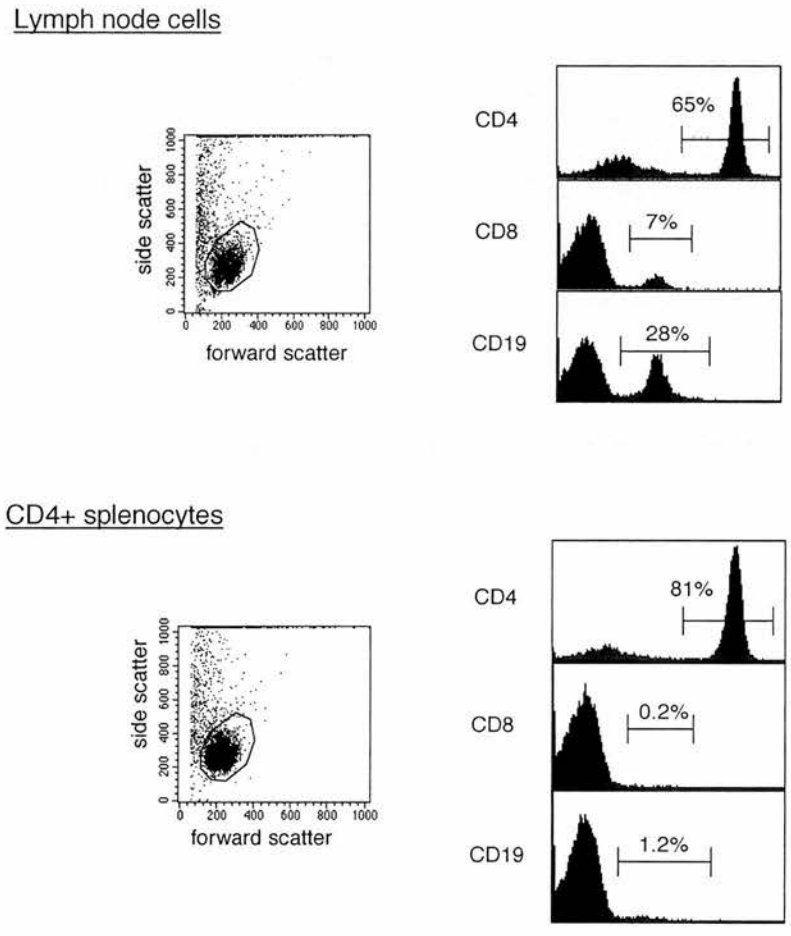

Figure 5.2 T cell purity before transfer. DO11.10 T cells were prepared for transfer either by pooling lymph nodes or by depleting splenocytes of CD8+ and MHCII+ contaminants with a MACS column. A sample was taken from each, stained with antibodies against CD4, CD8 and CD19 and analysed by flow cytometry. Forward and side scatter characteristics were used to define live cells, and the numbers give the percentage of these cells which fall within the marker shown.

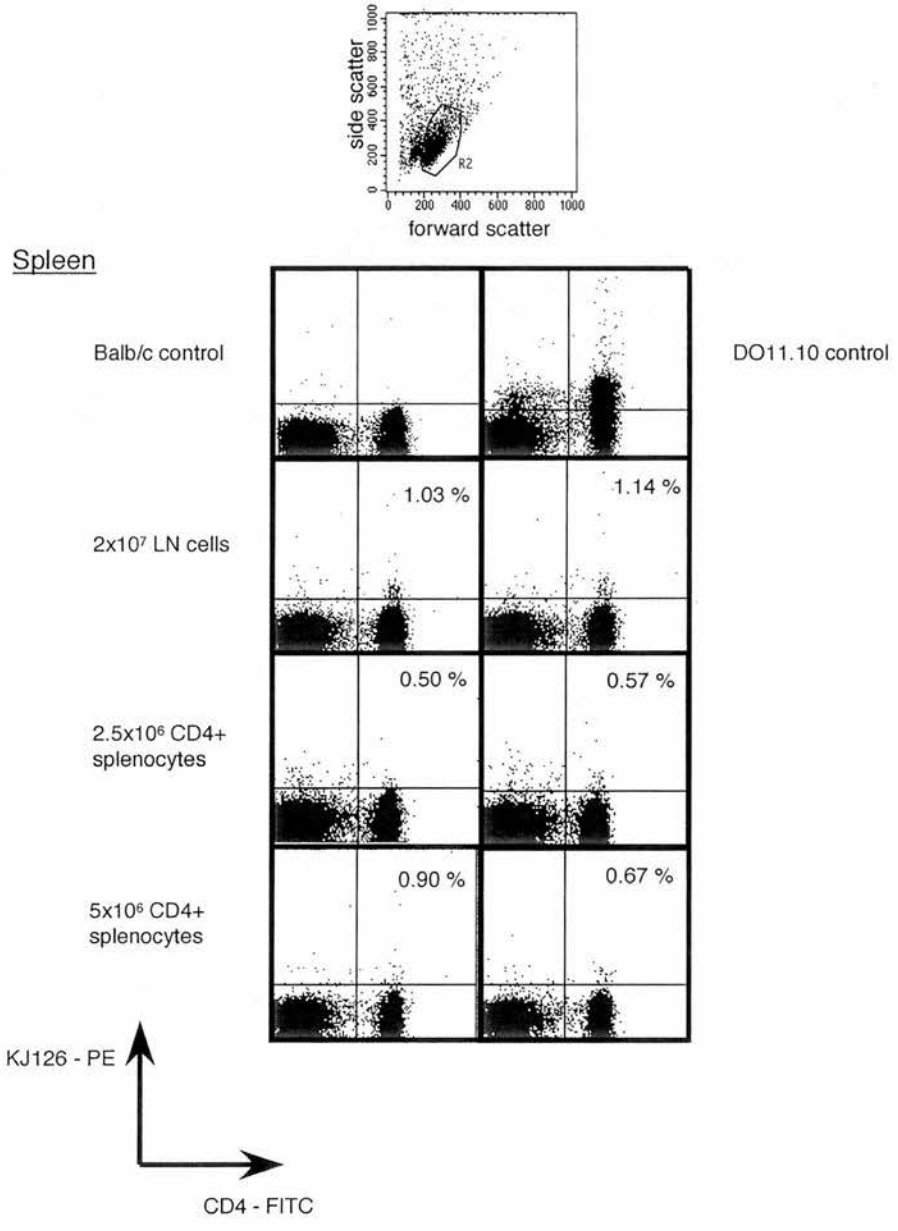

Figure 5.3 Detecting DO11.10 cells 24h after transfer, in the spleen. Mice received an adoptive transfer of different numbers of either lymph node cells or CD4+ enriched splenocytes. 24h later, their spleens were harvested, stained and analysed by flow cytometry. The numbers give the percentage of KJ126+ cells within the CD4+ population, using a live lymphocyte gate (R2) set on forward and side scatter characteristics as indicated at the top of the figure. The two plots represent the two mice in each group.

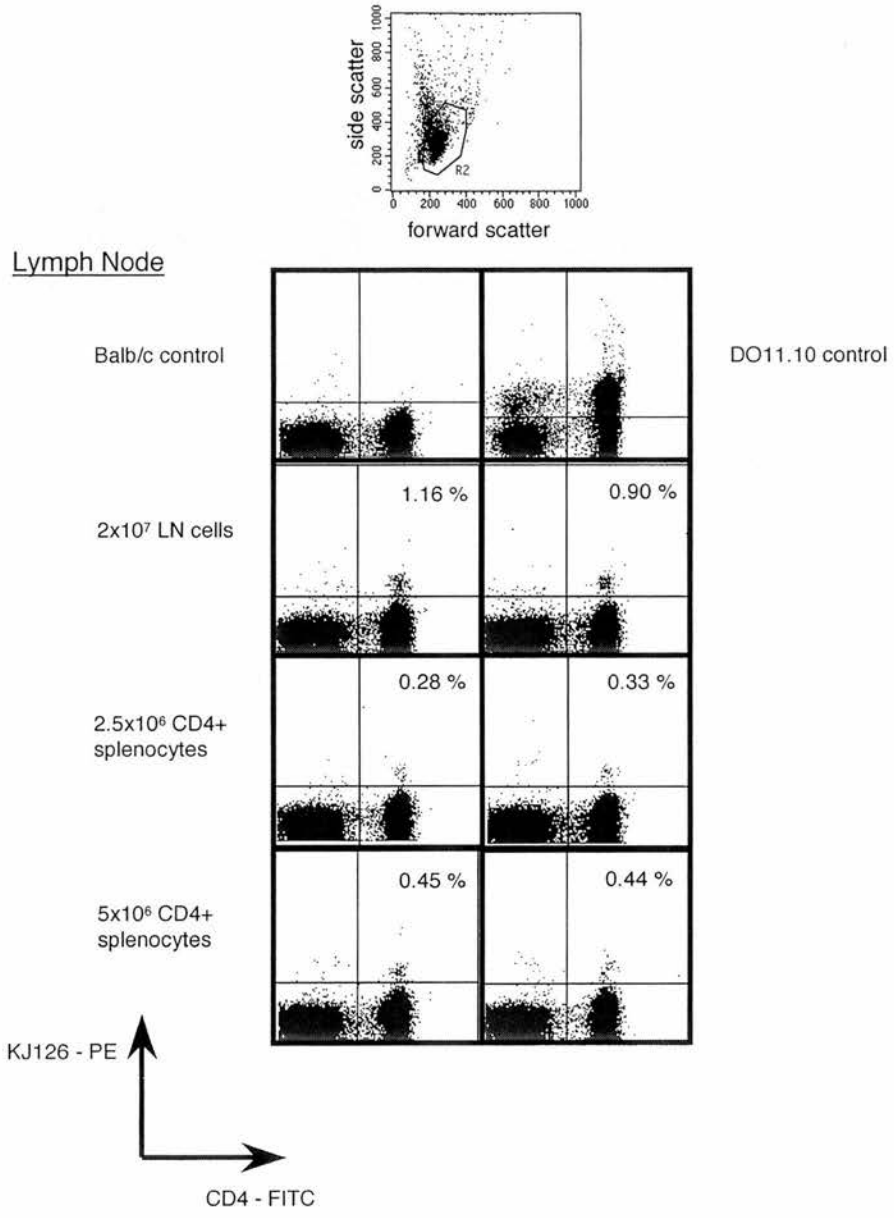

Figure 5.4 Detecting DO11.10 cells 24h after transfer, in the lymph nodes. As in figure 5.3, mice received an adoptive transfer of either lymph node cells or CD4+ enriched splenocytes and were sacrificed 24h later. Peripheral lymph nodes were collected, pooled and analysed by flow cytometry. The numbers give the percentage of KJ126+ cells within the CD4+ population, using a live lymphocyte gate (R2) set on forward and side scatter characteristics and shown at the top of the figure. The two plots represent the two mice in each group.

While the crude lymph node prep contained a lower percentage of CD4+ cells (61.3% ± 2.3, n=4) than the purified splenocytes (80.7% ± 3.0, n=5)(fig 5.2), the time needed to harvest and prepare the LNCs was considerably shorter. This had an edge of convenience, but it also meant that the cells were fresher and probably healthier when transferred into the recipient mice. The protocol of transferring $5x10^6$ LNCs into each recipient was therefore used in all subsequent experiments.

5.3.2 Without immunisation, survival of transferred cells is limited

To assess how long the transferred cohort of DO11.10 T cells lasted in the intact BALB/c host, mice were adoptively transferred with DO11.10 LNCs and killed at various times afterwards (fig 5.5). Early experiments had suggested that the transferred population was stable during the first 48h after transfer and indeed, the percentages seen 24h after the transfer of $5x10^6$ cells (although splenocytes rather than LNCs) in figs 5.3 and 5.4 are below those given for day 3 in fig 5.5. After day 3, however, this stability waned and the transgenic population declined steadily, becoming almost undetectable by day 28 post transfer. This was also reported by Jenkins and colleagues [437] and is probably due to homeostatic competition with

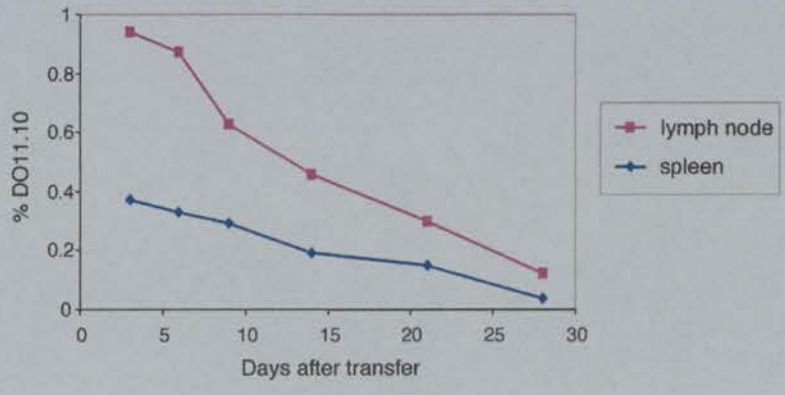

Figure 5.5 Loss of transferred T cells over time. DO11.10 lymph node cells were transferred into Balb/c recipients and two mice sacrificed at various times afterwards. The number of KJ126+ cells in their spleen and peripheral lymph nodes was measured by flow cytometry and is expressed as the percentage of live CD4+ cells in each tissue. Data is shown as the mean of the two samples at each timepoint.

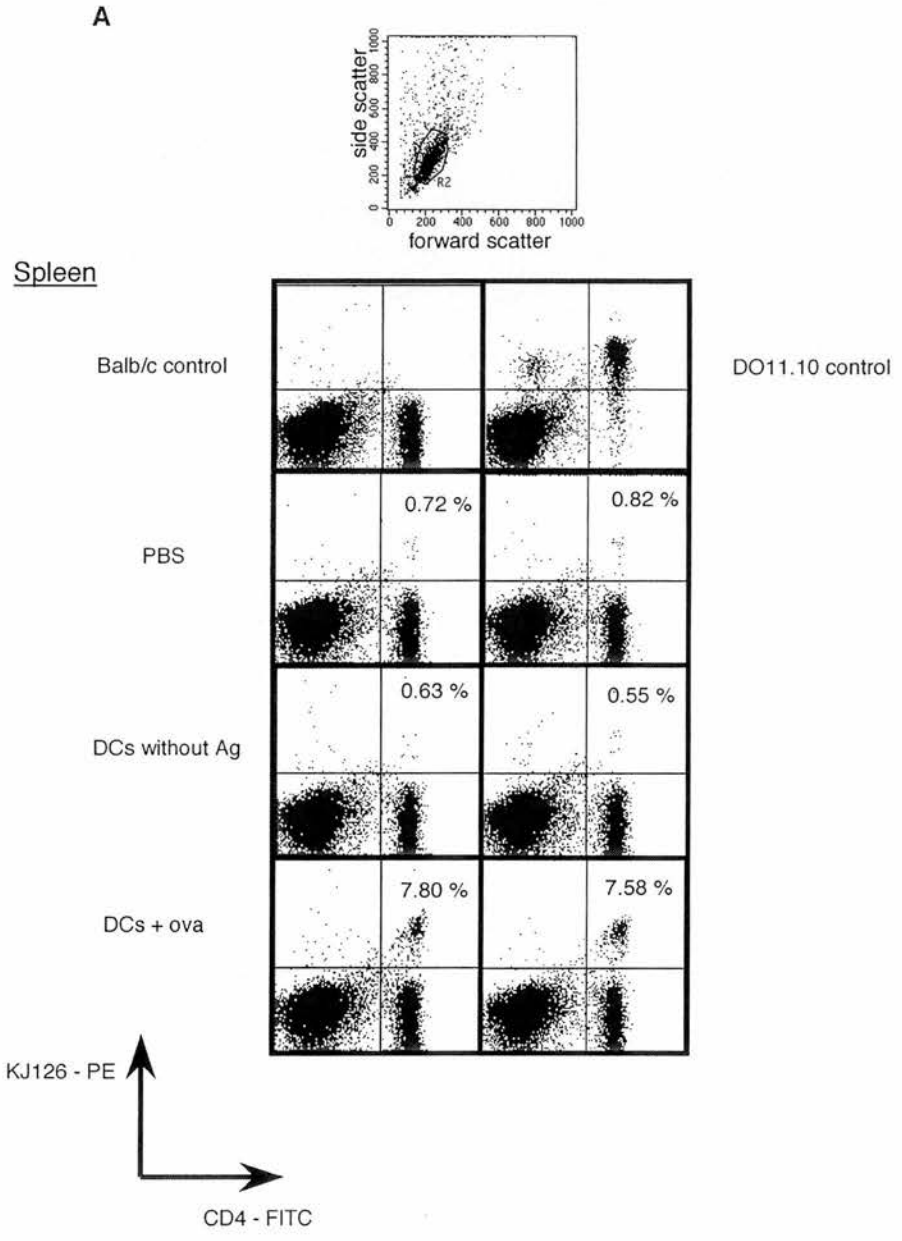

Figure 5.6A DO11.10 expansion in response to DC immunisation. DO11.10 LNC were transferred into mice 24 hours before immunisation with PBS, LPS-activated DCs or LPS-activated DCs pulsed with ova peptide. 5 days after immunisation, the mice were sacrificed and the percentage of DO11.10 cells in their spleens measured by flow cytometry. The raw data is shown here. The numbers give the percentage of KJ126+ cells within the CD4+ population, using the live cell gate shown at the top of the figure. The two plots represent the two mice in each group.

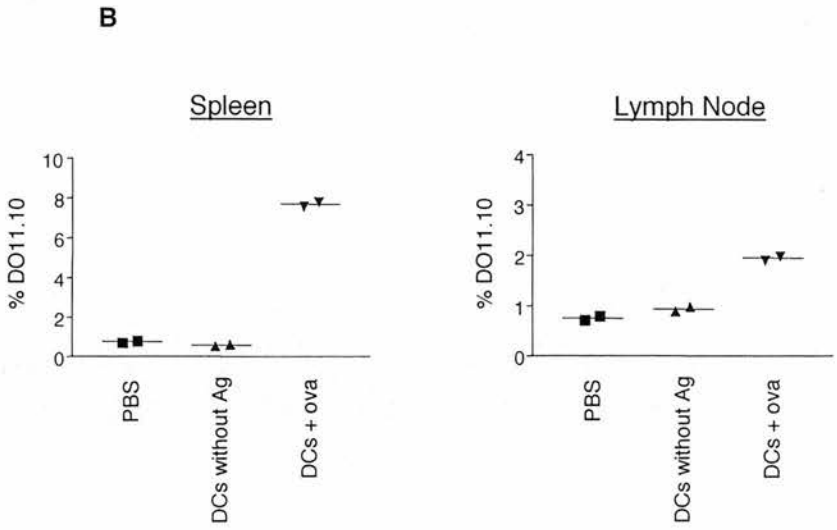

Figure 5.6B DO11.10 expansion in response to DC immunisation. These graphs illustrate the raw data shown in fig 5.6A, together with equivalent data from the lymph nodes of the same mice. The y-axis values give the percentage of KJ126+ cells within the live CD4+ population, and each point represents an individual mouse.

normal BALB/c T cells emerging from the thymus [445]. Practically, it meant that any immunisation of adoptively transferred mice had to be done within 48-72h of the initial transfer.

5.3.3 DC immunisation expands the transferred population

To confirm that DC immunisation could elicit an antigen specific immune response, immature DCs were harvested at day 7 of culture and stimulated with LPS for 6h. Half the cells were then pulsed with ova peptide and half maintained in medium alone. The two populations were injected i.v. into mice that had been adoptively transferred with DO11.10 LNCs 24h earlier. The mice were sacrificed 5 days later and the percentage of CD4+ KJ126+ transgenic cells in their spleens and lymph nodes measured by flow cytometry. The raw data is shown in fig 5.6A and summarised in fig 5.6B. In both tissues, there was a clear and antigen specific expansion of the DO11.10 cells in response to the peptide-loaded DCs. Mice

receiving unpulsed DCs and those mock-injected with PBS displayed a low level of KJ126 staining consistent with unimmunised transfers 6d after delivery of the T cells (see fig 5.5).

5.3.4 The primary response peaks at day 5

Pulendran *et al.* [166] and Maldonado-Lopez *et al.* [152] recommended measuring the immune responses triggered by antigen-loaded DCs 4-5 days after immunisation. Figure 5.7 confirmed that day 5 also caught the peak of the primary response in the DC-immunised adoptive transfers used here. The data also revealed an interesting disparity between the two lymphoid tissues studied. Systemic administration of antigen-loaded DCs induced more extensive expansion of the DO11.10 population in the spleen than in the lymph nodes (figs 5.6 and 5.7).

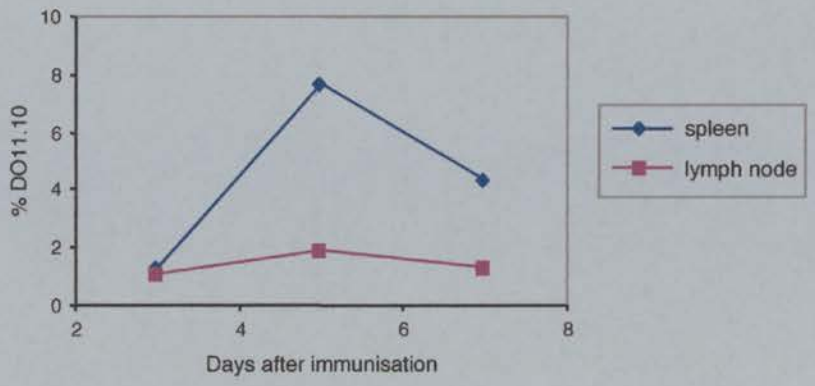

Figure 5.7 The primary response peaks at day 5. Adoptive transfers were immunised with LPS-activated DCs pulsed with ova peptide and two mice sacrificed at 3, 5 and 7 days later. The number of KJ126+ cells in their spleen and peripheral lymph nodes was measured by flow cytometry and is expressed as the percentage of live CD4+ cells in each tissue. Data is shown as the mean of the two samples at each timepoint.

5.3.5 IL-10 treated DCs stimulate limited T cell expansion in vivo

Having established that DO11.10 T cells could be transferred successfully and would respond to ova peptide, I wanted to test whether DCs activated by LPS in the presence of IL-10 would generate the same degree of T cell expansion as DCs given LPS alone. Both DC populations were stimulated for 6h, pulsed with ova peptide and injected i.v. into mice adoptively transfered with DO11.10 LNCs 24h earlier. The percentage of CD4+ KJ126+ T cells in their spleens was measured 5d later by flow cytometry. Unstimulated DCs induced a moderate T cell response, but activating them with LPS significantly enhanced the T cell expansion ($P<0.0001$, fig 5.8). When the LPS was given together with IL-10, this increase disappeared ($P=0.03$, fig 5.8). This is very similar to the effect of IL-10 treated DCs seen *in vitro* (compare with fig 4.2).

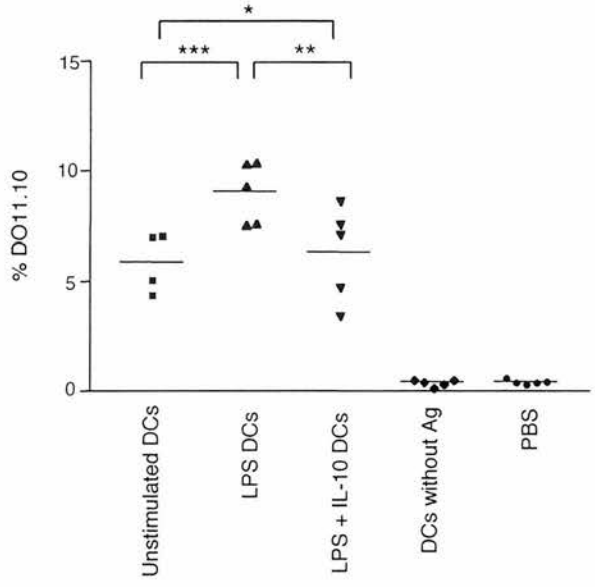

Figure 5.8 IL-10 treated DCs stimulate a limited T cell response *in vivo*. Mice received an adoptive transfer of DO11.10 LNC 24h before being immunised with unstimulated DCs (medium alone), DCs activated with LPS for 6h or DCs stimulated with LPS in the presence of IL-10, all pulsed with ova peptide. As negative controls, mice were included that had been immunised with DCs not pulsed with antigen and that had been mock-injected with PBS alone. Mice were sacrificed 5 days after immunisation and the number of KJ126+ cells in their spleens measured by flow cytometry. Data is expressed as the percentage of KJ126+ cells within the live CD4+ population and each point represents an individual mouse. The graph is representative of seven independent experiments, each using 4-5 mice per group. *, $P = 0.044$; **. $P = 0.031$; ***, $P < 0.0001$; using an unpaired students t-test on data compiled from all experiments.

5.3.6 Background proliferation

As *in vitro*, the limited T cell expansion generated by IL-10 treated DCs could reflect either inefficient priming or an active suppression of the T cell response. To distinguish the two possibilities, mice that had received an adoptive transfer of DO11.10 LNCs were immunised with the different DC populations and their spleens harvested at day 5, the peak of the primary response, and restimulated *in vitro*. Unfortunately proliferation in these cultures proved resoundingly non-specific: there was no reduction in counts as the ova peptide was titrated out and even wells without peptide scored upwards of 70000 cpm (fig 5.9A). Importantly, the spleens of mice given mock injections of PBS proliferated very little while those from mice immunised with unpulsed DCs showed the same high background as the three experimental groups. This suggested that the problem was not an isolated feature of the *in vitro* culture, but more likely a secondary response to another antigen unwittingly loaded onto the DCs alongside the ova peptide.

Serum antigens were obvious candidates, especially as the emergence of high background proliferation coincided with a change in serum supplier. Despite extensive investigation of the batch variability of commercial mouse serum, though, the explanation remained elusive. Mismatching the sera and even the media used for the preparation of the DCs with that involved in the restimulation culture was unsuccessful. There was no evidence of superantigen contamination of media or other reagents and indeed simple *in vitro* proliferation assays with DO11.10 T cells remained ova-dependent. One possibility was that the tail vein injection of transgenic T cells just 24 hours before delivering DCs into the same site created a damaged or inflammatory environment that at least contributed to the unexpected response. Fig 5.9B suggests otherwise. These mice were immunised without any prior adoptive transfer and yet their background proliferation remained high. The extent of the problem was illustrated by CFSE labelling of the restimulation cultures (fig 5.10). Two colour staining with KJ126 allowed the ova-specific DO11.10 cells to be distinguished from the BALB/c majority, and neatly demonstrated that while both the expansion of the DO11.10 cells *in vivo* and their division *in vitro* required

ova peptide, this response was dwarfed by the massive proliferation of the KJ126- population.

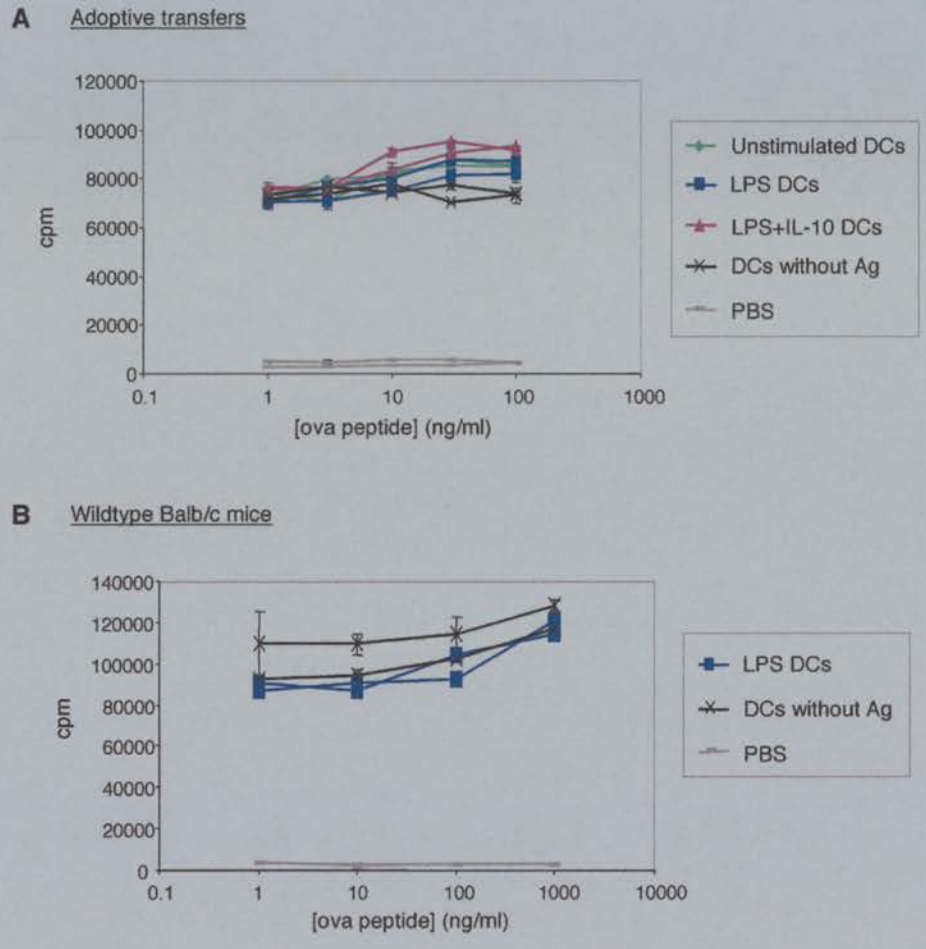

Figure 5.9 High background proliferation. (A) Mice received an adoptive transfer of DO11.10 LNCs and were immunised 24h later with DCs pulsed with ova peptide. Their spleens were harvested 5d after immunisation and plated in vitro with graded doses of antigen. (B) Wildtype mice were immunised with DCs without any prior adoptive transfer. Their spleens were harvested at day 5 as above. In both cases, proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM and the two lines per immunisation represent the two mice per group.

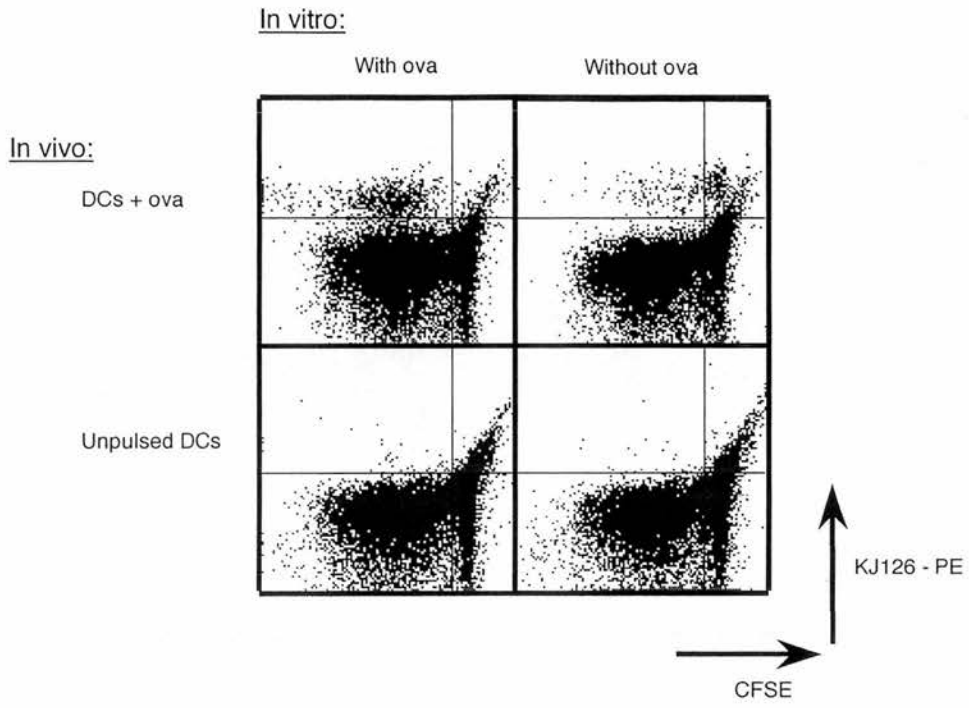

Figure 5.10 Non-specific proliferation illustrated by CFSE staining. Mice received an adoptive transfer of DO11.10 LNCs and were immunised 24h later with LPS-stimulated DCs either coated with ova peptide or left without antigen. Their spleens were harvested 5d after immunisation and labelled with CFSE before being cultured *in vitro* for a further 4 days. These cells were counterstained with KJ126-PE and their fluorescence assessed on a flow cytometer. Dividing DO11.10 cells fall into the top left quadrant.

5.3.7 Restimulation at day 5

The problem of high background proliferation in *in vitro* restimulation cultures was surprisingly solved by moving the mouse serum from the freezer to the fridge for routine storage. The beauty of the peptide dependent responses seen at last in fig 5.11 therefore masked the disappointing similarity of the experimental groups. Mice that had been adoptively transferred with a cohort of DO11.10 LNCs were immunised with DCs that were attempted in figure 5.9, adoptive transfers had been immunised with DCs that were either unstimulated, activated with LPS or treated with LPS and IL-10 together. Their spleens were harvested at day 5 and cultured with peptide. Both the PBS and unpulsed DC controls were negative, but all three

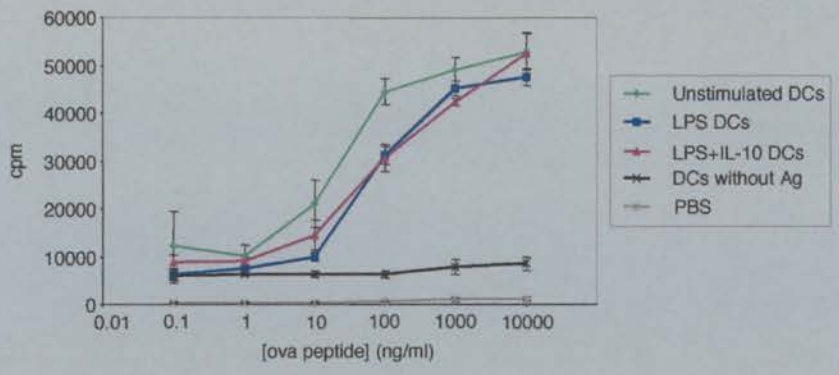

Figure 5.11 Ex vivo restimulation at day 5. Mice received an adoptive transfer of DO11.10 LNCs 24h before being immunised with untreated DCs, LPS-activated DCs or DCs stimulated with LPS+IL-10, all coated with ova peptide. Mice that received an adoptive transfer but were immunised with LPS-activated DCs without antigen or a mock-injection of PBS were included as negative controls. Mice were sacrificed 5d after immunisation and their spleens pooled within groups and plated for *in vitro* culture with graded doses of ova peptide. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture and data is shown as the mean of triplicate wells ± SEM. The graph is representative of two independent experiments, each with 5 mice per group.

DC treatments resulted in equivalently strong *in vitro* recall responses. Given the higher percentage of DO11.10 T cells found in the spleens of mice immunised with LPS-activated DCs on day 5 (see fig 5.8), their proliferation per KJ126+ cell at this stage was in fact lower than that achieved by either unstimulated or IL-10 treated DCs.

5.3.8 Secondary expansion in vivo is small

The similarity of the proliferation curves in fig 5.11 was perplexing. While it was certainly possible that the limited T cell expansion generated by IL-10 treated DCs was due to inefficient priming, leaving the majority of the DO11.10 cells as fully

capable of responding to antigenic challenge as any other naïve population, I still expected immunisation with LPS DCs to result in a stronger, secondary-type response to the *in vitro* restimulation. This was not the case. One possible explanation was that by harvesting and restimulating the spleens at the peak of the primary response, the T cells activated by the DC immunisation were left refractory to the second stimulation. The proliferation seen in fig 5.11 might then be a primary response of DO11.10 cells left unactivated by the original, *in vivo* challenge.

To address this, the time between primary and secondary challenge was increased and, importantly, the restimulation was also delivered *in vivo*. Mice that had been adoptively transferred with DO11.10 LNCs were immunised with DCs that had been cultured in medium alone, with LPS or with LPS and IL-10 together. 7 days later, all three groups were reimmunised with LPS-activated DCs. To measure their T cell expansion in response to the second challenge, mice were either sacrificed or bled 3 days later, on day 10. The percentage of DO11.10 cells in their CD4+ pool was assessed by flow cytometry, and the result is shown in fig 5.12. Mice that were

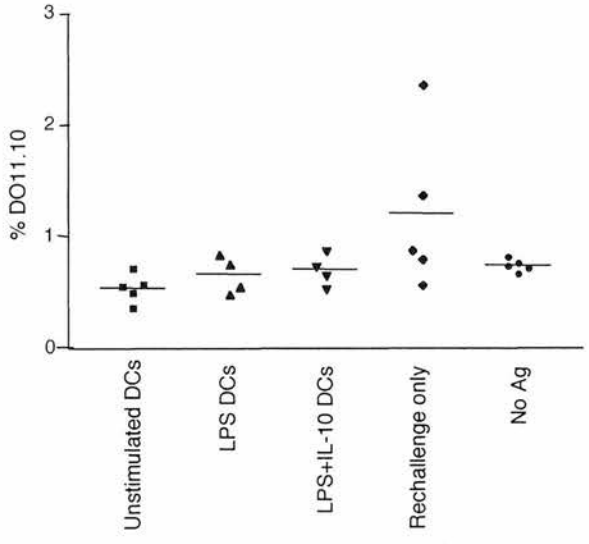

Figure 5.12 T cells show little expansion to secondary challenge *in vivo*. Adoptive transfers were immunised with untreated DCs, LPS-activated DCs or DCs stimulated with LPS+IL-10, all coated with ova peptide. Transfers receiving LPS-activated DCs without antigen were included as a negative control. Seven days later, mice were rechallenged with LPS-activated DCs pulsed with peptide. The negative control group again received DCs without antigen (No Ag), and a positive control group was set up with an adoptive transfer 24h before being given only the same LPS DC + ova 'rechallenge'as the experimental mice (Rechallenge only). Mice were sacrificed 3d after rechallenge,on day 10 of the experiment, and the number of KJ126+ cells in their spleens measured by flow cytometry. Data is expressed as the percentage of KJ126+ cell within the live CD4+ population and each point represents and individual mouse. The graph is representative of five independent experiments, each using 4-5 mice per group. There is no significant difference between any of the experimental groups, using an unpaired students t-test.

immunised twice with unpulsed DCs illustrated the background level of DO11.10 chimaerism established by the adoptive transfer. The positive control group, animals that were given the second round of LPS-stimulated DCs as a primary challenge, showed some T cell expansion but at only 3 days after immunisation, they were still in the early stages of their response. In contrast to the large secondary expansion I had anticipated from at least those immunised with LPS DCs, all three experimental groups showed very little increase above background. Although initially surprising, this inhibition of secondary proliferation was also reported in the original description of the adoptive transfer technique [195] and has recently been shown to be imposed by external factors, perhaps persisting antigen, within the immunised host [383].

5.3.9 Restimulation at day 17

To overcome the lack of T cell expansion, Merica *et al.* recommended measuring responses to *in vivo* rechallenge by purifying the DO11.10 population and re-transferring them into a fresh, unimmunised host [383]. As an alternative approach, here the experimental mice were kept until day 17, 10d after the second DC injection, and then their spleens taken for *in vitro* restimulation. The delay until day 17 ensured that the DO11.10 expansion had returned to baseline in all groups (fig 5.13A), and was designed to avoid any refractory behaviour from the responding T cells. The resulting proliferations are shown in fig 5.13B. Differences between the groups were small, but on the two separate occasions that the experiment was performed, splenocytes from mice originally immunised with IL-10 treated DCs proliferated less than those from mice given either unstimulated or LPS-activated DCs. The initial interaction with DCs stimulated in the presence of IL-10 appeared to inhibit the T cell response to subsequent challenge.

This result was even clearer when the *in vivo* rechallenge was given as a s.c. injection of peptide in CFA, in place of the second dose of antigen-loaded DCs (fig 5.14). As above, adoptive transfers were immunised with the different DC populations, rechallenged on day 7 and sacrificed on d17. Cells from the lymph node which

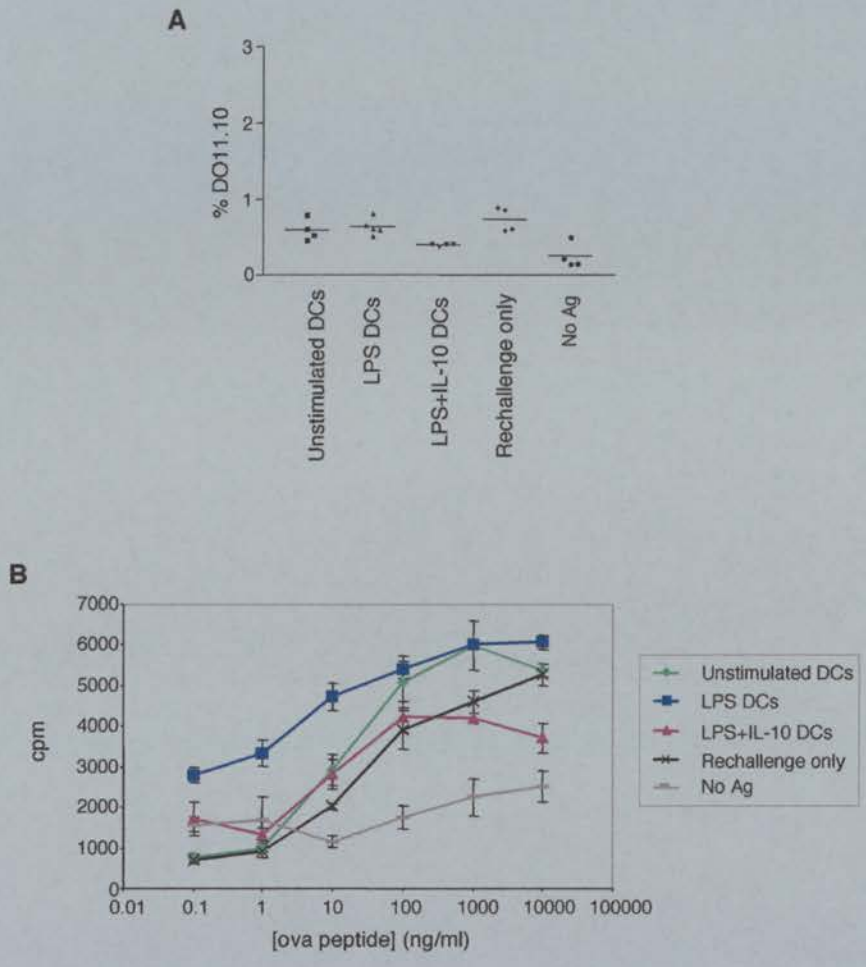

Figure 5.13 In vivo rechallenge with LPS DCs. As in figure 5.10, adoptive transfers were immunised with untreated DCs, LPS-activated DCs or DCs stimulated with LPS+IL-10, all coated with ova peptide, and rechallenged 7d later with LPS DCs pulsed with peptide. The positive control group (Rechallenge only) received an adoptive transfer 24h before being given the same DC + ova as the experimental mice. The negative controls (No Ag) were given LPS DCs without antigen at both immunisation and rechallenge. Mice were sacrificed 10d after rechallenge, on day 17 of the experiment. (A) The number of KJ126+ cells in their spleens was measured by flow cytometry. Data is expressed as the percentage of KJ126+ cell within the live CD4+ population and each point represents an individual mouse. There is no significant difference between experimental groups, using an unpaired student's t-test. (B) The remaining splenocytes were pooled within groups and plated for *in vitro* culture with graded doses of ova peptide. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM. Both panels are representative of two independent experiments, each using 4-5 mice per group.

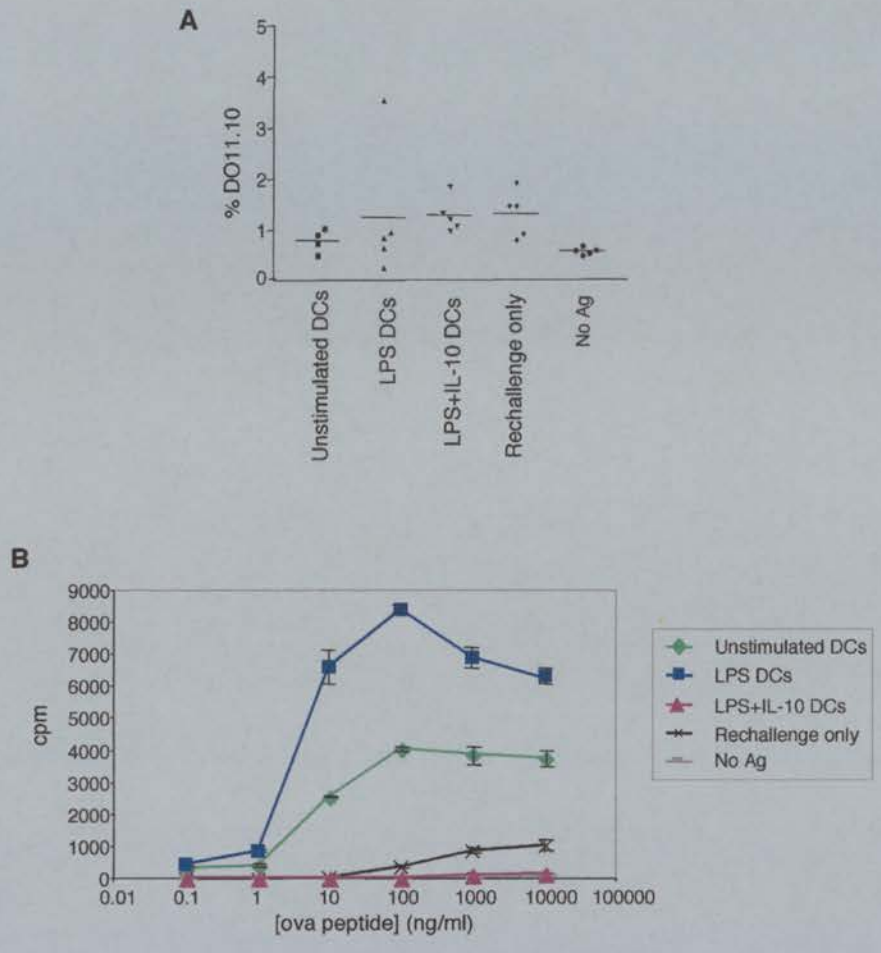

Figure 5.14 In vivo rechallenge with peptide in CFA. Adoptive transfers were immunised with untreated DCs, LPS-activated DCs or DCs stimulated with LPS+IL-10, all coated with ova peptide, and rechallenged 7d later with ova in CFA. The positive control group (Rechallenge only) received an adoptive transfer 24h before being given the same ova in CFA as the experimental mice. The negative controls (No Ag) were given LPS DCs without antigen at immunisation and PBS in CFA at rechallenge. Mice were sacrificed 10d after rechallenge, on day 17 of the experiment. (A) The number of KJ126+ cells in the draining lymph nodes was measured by flow cytometry. Data is expressed as the percentage of KJ126+ cell within the live CD4+ population and each point represents an individual mouse. There is no significant difference between groups, using an unpaired student's t-test. (B) The remaining lymph node cells were pooled within groups and plated for *in vitro* culture with graded doses of ova peptide. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM. Both panels are representative of two independent experiments, each using 4-6 mice per group.

drained the site of CFA injection were stained to measure the percentage of DO11.10 T cells present and plated with peptide to assess their proliferative response to restimulation. Again the T cell expansion triggered by the *in vivo* rechallenge was limited (fig 5.14A), but this had the advantage of ensuring a similar number of transgenic T cells in each well of the restimulation assay. Lymph node cells from the different groups of mice showed distinct proliferative responses, however (fig 5.14B). LNCs from adoptive transfers first immunised with LPS activated DCs proliferated strongly, typical of a primed response. Those from transfers immunised with unstimulated DCs also responded strongly; the discrepancy between this and that of the LPS DCs might be explained by the slight difference in percentage of DO11.10 cells present (see fig 5.14A). Importantly though, very little proliferation was achieved by LNCs from mice first given DCs stimulated with LPS and IL-10 together. These DCs clearly inhibited subsequent T cell responses.

5.3.10 Restimulation in bone marrow chimaeras

Although still in need of repetition, the data shown in figs 5.13 and 5.14 together suggest that immunisation with DCs activated in the presence of IL-10 can be sufficient to tolerise a cohort of specific T cells *in vivo*. An exciting question was then whether these T cells were simply inactivated, perhaps anergised, or whether an active suppression was involved. One way to address this would be to purify the non-proliferative T cells, transfer them into a fresh BALB/c host at the same time as another cohort of naïve DO11.10 LNCs, and test whether they can inhibit expansion of the naïve cells. An alternative approach, and the one adopted here, is to immunise and rechallenge a group of mice that are chimaeric for the DO11.10 transgene at the level of their bone marrow rather than just in the periphery, as in the adoptive transfers. These chimaeric mice were created by lethally irradiating BALB/c recipients before reconstituting them with a mixture of BALB/c and DO11.10 bone marrow. The ratio of BALB/c to DO11.10 cells was chosen to ensure that 1-2% of mature T cells carried the Transgenic TCR (Simon Fillatreau, ICAPB, University of Edinburgh, personal communication). Because these mice are bone marrow

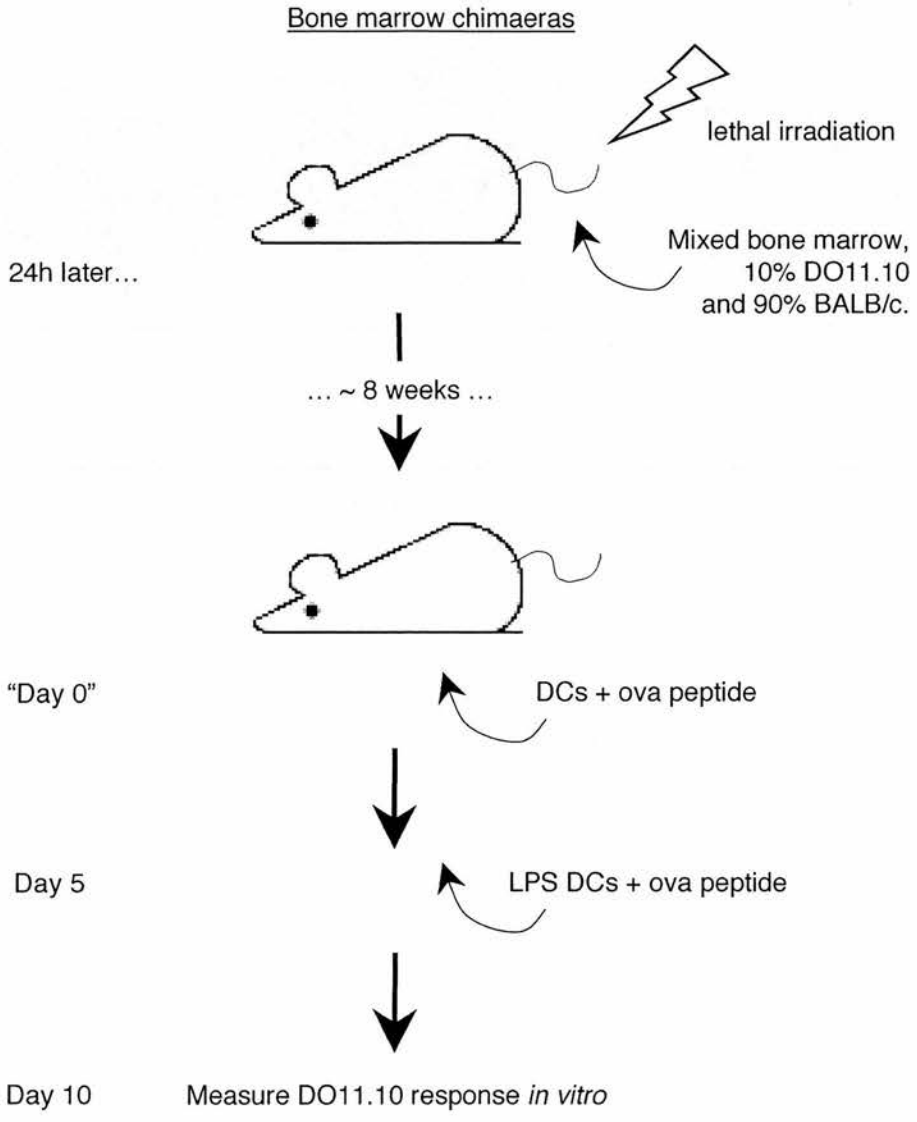

Figure 5.15 Outline of bone marrow chimaera experiment. BALB/c hosts were lethally irradiated and, 24h later, injected with bone marrow from both BALB/c and DO11.10 mice, combined at a 9:1 ratio. The mice were left 8 weeks to allow reconstitution of the immune system. They were then immunised with different DC populations, each coated with ova peptide. 5 days later, they were rechallenged *in vivo* with LPS-activated DCs also pulsed with peptide. Mice were sacrificed at day 10, and their response to ova measured in an *in vitro* assay.

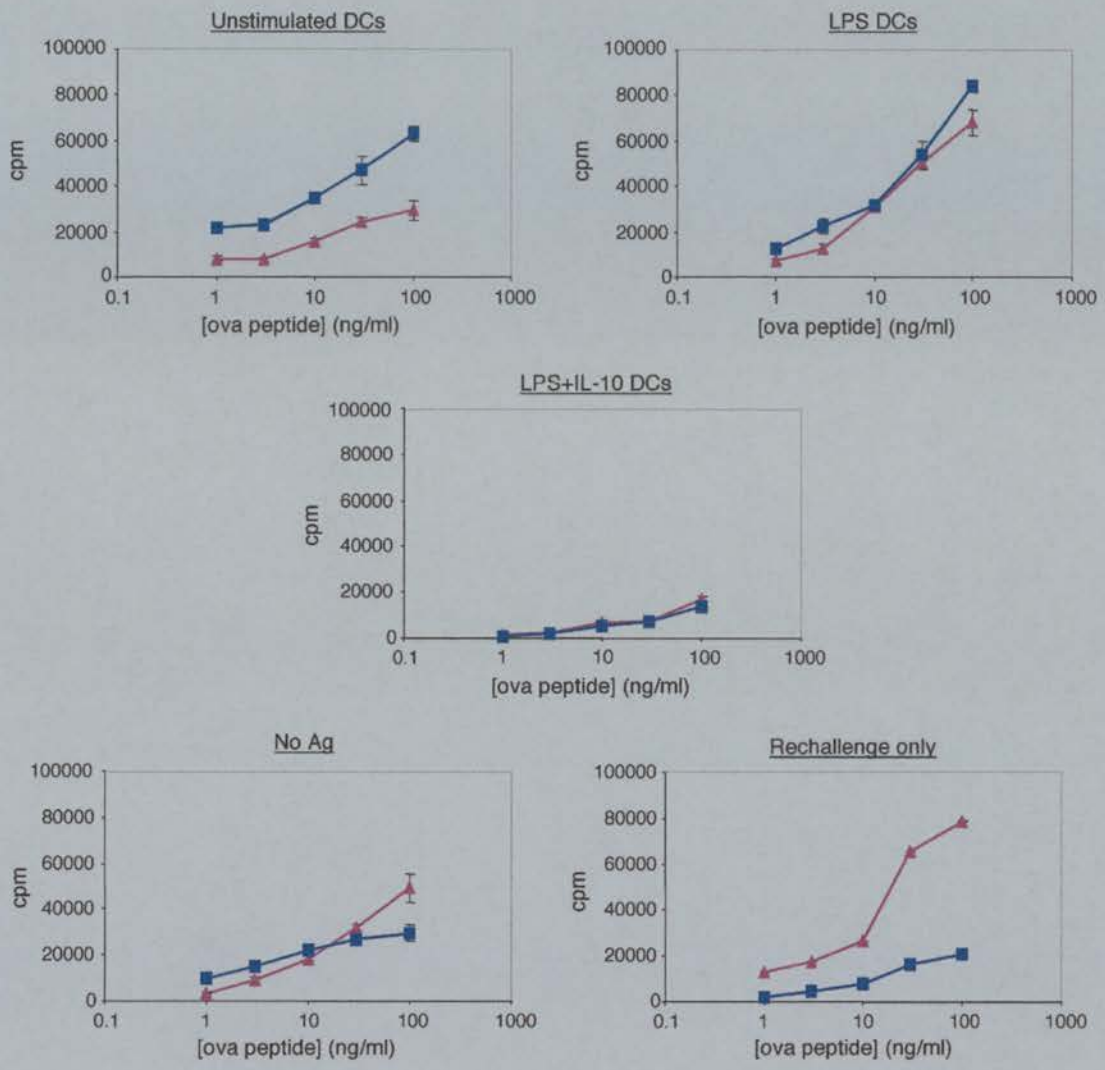

Figure 5.16 In vivo rechallenge in bone marrow chimaeras. Chimaeric mice were created by reconstitution of an irradiated BALB/c host with 90% BALB/c and 10% DO11.10 bone marrow (see section 2.10 and fig 5.15). These mice were immunised with untreated DCs, LPS-activated DCs or DCs stimulated with LPS+IL-10, all coated with ova peptide, and rechallenged 5d later with LPS DCs pulsed with peptide. The positive control group (Rechallenge only) were immunised with LPS DC + ova only on day 5. The negative controls (No Ag) were given LPS DCs without antigen at both immunisation and rechallenge. Mice were sacrificed 5d after rechallenge, on day 10 of the experiment. Their spleens were harvested and plated for *in vitro* culture with graded doses of ova peptide. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM and the two lines represent the two mice in each group.

chimaeras, an initial injection of IL-10 treated DCs may inactivate the population of DO11.10 T cells present at that time but more Transgenic cells will emerge from the thymus before administration of the *in vivo* rechallenge. Again, any suppression of these naïve cells would suggest an active role for the originally tolerised T cells.

Chronologically this was the first *in vivo* experiment performed; bone marrow chimaeras were made routinely in the lab while the adoptive transfer system took time to establish. As such, the timing of the successive immunisations lacked some of the refinement later used in the experiments described above. Chimaeric mice were immunised with DCs that were either unstimulated or activated with LPS alone or in combination with IL-10, and then, five days later, were rechallenged with a second injection of LPS-activated DCs. After a further five days, on day 10, the mice were sacrificed and their splenocytes cultured in the presence of ova peptide for an *in vitro* restimulation assay (fig 5.15). The results are shown in fig 5.16. As in the adoptive transfer system, the strongest proliferation was shown by splenocytes from chimaeras originally immunised with LPS-activated DCs. If the first immunisation was given with unstimulated DCs, the proliferation was less pronounced. IL-10 treated DCs resulted in a marked inhibition of the restimulation response, leaving a level of proliferation below even that of the negative controls (fig 5.15). This suggests that DCs activated in the presence of IL-10 might be able to induce an active suppression of the immune response. It remains very preliminary, however. The experiment was done once with only two mice per group, and the poverty of this approach was demonstrated by the two positive controls, only one of which responded (fig 5.15).

5.4 Discussion

The combination of DO11.10 adoptive transfers and the KJ126 monoclonal antibody provides a powerful tool for investigating immune responses in a near-physiological setting [437]. Here the system was exploited to examine the impact of IL-10 treated DCs on a T cell response *in vivo*. The data suggest that DCs activated in the presence of IL-10 elicit a limited T cell expansion that confers a lasting suppression,

leaving the T cells hyporesponsive to subsequent challenge. These results reinforce those generated *in vitro* in chapter 4, and suggest an exciting therapeutic promise.

First, however, initial experiments confirmed that the adoptive transfer system could work here as it does elsewhere [195, 443, 444]. The original description of the technique [195] reported a frequency of ~0.5% transgenic T cells in the spleen and lymph nodes following transfer of $2.5x10^6$ T cells, and the percentages achieved here were similar (figs 5.3 and 5.4). The transferred cells were shown to proliferate strongly in response to a primary immunisation with antigen loaded DCs (fig 5.6), but surprisingly weakly to a secondary challenge (fig 5.12). This was also detailed by Jenkins's group [383] and it perhaps challenges a common perception that part of the memory phenotype is an inherent propensity for rapid division upon stimulation [446]. Merica *et al.* demonstrated that transferring the T cells into a naïve host or delaying the rechallenge by several weeks, presumably allowing residual peptide:MHC complexes to disappear, resulted in a return to the slower but more expansive proliferation typical of a primary response [383]. This was associated with a reversion to a 'naïve' surface phenotype with high levels of CD45RB [383, 447]. Their conclusion was that persistence of CD4+ mediated immunity after elimination of the original antigen is due to the presence of both specific antibodies, which efficiently target fresh antigen to APCs [448, 449], and an expanded population of specific T cells, even though these share some functional characteristics with naïve cells [383, 450]. This has two main implications for the data presented here. The first is reassuring: the lack of T cell expansion seen in response to secondary challenge is not a flaw in the system and should perhaps have been expected. The second is intriguing: if CD4+ T cell immunity is at least partly due to environmental factors such as persistent antigen or specific antibodies, rather than being an inherent property of the T cells, is the same also true of CD4+ T cell tolerance?

Another interesting point to emerge from these early experiments was the disparity in the degree of expansion of DO11.10 cells in spleen and lymph node, illustrated most clearly in figs 5.6 and 5.7. The data is presented as a percentage of CD4+ cells, to account for the different number of T cells in each tissue, so the explanation is more

complex than this. The DCs were administered systemically, injected i.v. into the tail vein, so the spleen is perhaps a natural destination. The transferred T cells were delivered in the same way, however, and before immunisation there was no such distinction between tissues. Naïve T cells express homing receptors to carry them into lymph nodes, such as L-selectin, CD62L, which facilitates passage through high endothelial venules [451]. In contrast, the chemokine receptors on mature DC are designed to take them into LNs from the lymph, not blood [398, 452], so systemic delivery may well by default cause their accumulation in the spleen. Labelling the T cells or the DCs with tracking dyes before injection would be revealing.

Although the primary T cell expansion generated by IL-10 treated DCs was concentrated in the spleen, the inhibition of secondary responses was systemic, evident even when the rechallenge was given as a s.c. immunisation in the thigh (fig 5.14). This presumably reflects the circulation of effector T cells. Interestingly, while the suppression was apparent in splenic proliferation assays, it was more pronounced in lymph node cultures (compare figs 5.13 and 5.14). Different degrees of tolerance in spleen and lymph node have been reported elsewhere [453], but the reason for these discrepancies is uncertain. The cellular composition of the spleen and lymph node is quite different [454], but the predominance of B cells in the spleen might be expected to encourage tolerance there [455, 456]. The drainage systems that deliver antigen to the two tissues are also distinct, but again this offers little explanation. Systemic antigens often elicit tolerance [195, 457] and many localised, peripheral infections require strong immunity to clear them [458, 459]. It may reflect the design of the experimental systems rather than a physiological reality, but either way it remains puzzling.

The induction of tolerance by IL-10 treated DCs is not a new idea. Enk *et al.* first described their ability to anergise a murine Th1 clone in 1993 [351]. Steinbrink *et al.* repeated this data using both human CD4+ [205] and CD8+ [460] cells, and more recently the same group have attributed a suppressor function to these anergic cells [349]. McBride *et al.* also used cocultures of human DCs and CD4+ T cells to demonstrate that stimulation of DCs in the presence of IL-10 inhibits T cell

responses to subsequent challenge [461]. The data presented here have the distinction of illustrating that the same suppression is possible *in vivo*.

The other key difference between these two sets of data is in the interpretation of the status of the IL-10 treated DCs. All of these reports describe the effect of IL-10 as an inhibition of maturation, so while their work is restricted to an *in vitro* setting, it draws strength from the use of immature DCs *in vivo*. Dhodapkar *et al.* injected autologous, immature DCs into two human volunteers and saw both the inhibition of CD8+ cytolytic activity and the emergence of IL-10 producing CD4+ cells [325]. In mice, immature DCs have been shown to temporarily [462] or indefinitely [463] prolong cardiac allograft survival. The *in vitro* data presented in chapters 3 and 4 suggest that DCs stimulated in the presence of IL-10 are not immature, however. They downregulate antigen uptake and rapidly increase their expression of MHC and B7. Even at the peak of their activation, though, IL-10 treated DCs fail to stimulate T cell proliferation. Their activated state gives them a theoretical advantage over immature cells: it implies a greater functional stability, and hence a smaller risk of an unwanted immune response. Interestingly, the DCs that enabled seemingly permanent acceptance of the murine heart transplant were generated by culture in very low concentrations of GM-CSF [463]. The stringency of these conditions resulted in considerable apoptotic death, and the uptake of apoptotic cells by DCs has been shown to induce an actively tolerogenic phenotype [464].

The difficulty with arguing that the suppression of T cell responses by IL-10 DCs *in vivo* happens despite or even because of their high level expression of MHC and B7, is one of timing. *In vitro*, the DCs were stimulated for 6h, pulsed with peptide antigen and immediately introduced to purified transgenic T cells (chapter 4). The opening conversation between the DCs and responding T cells was therefore very likely to occur while the IL-10 treated DCs were still phenotypically activated. In contrast, the protocol used for the *in vivo* experiments meant that DCs were expressing high levels of MHC and B7 as they were injected. The time needed to travel to secondary lymphoid tissue and then to encounter a specific T cell might mean that, at the point of T cell interaction, the IL-10 treated DCs had passed their peak of activation and had begun to downregulate their surface markers (see fig 3.7).

Labelling DCs with fluorescent dye before injection and monitoring their arrival in the spleen or lymph nodes would allow a relevant assessment of the timing involved. It may be surprisingly quick. Ingulli *et al.* injected ova peptide s.c. into the back of mice and used microscopy to visualise physical contact between DCs that acquired the antigen in vivo and T cells in the draining lymph node. Interaction began 6 hours after immunisation and peaked at 12h, a time window that closely matched the production of IL-2 by the T cells [384]. Merica et al. measured IL-2 production by naïve DO11.10 T cells in lymph nodes within 2h of i.v. injection of ova peptide, and maximal levels were seen at 6h. In antigen experienced T cells, this peak occurred at just 1.5h after injection [383]. The high levels of MHC and B7 on IL-10 treated DCs early after LPS stimulation may still be relevant to the *in vivo* experiments described here. The absence of cytokine production by these DCs, the presence of actively inhibitory signalling or perhaps the premature termination of T cell stimulation might explain the lack of T cell expansion and the consequent suppression of secondary responses observed (see figs 5.8, 5.13 and 5.14, and the discussion in chapters 3 and 4). In support, Taams *et al.* have demonstrated that anergic T cells can convert an APC into one which is unable to stimulate proliferation in normal responder cells and, importantly, that this happens without any reduction in surface levels of MHCI, MHCII, B7.1 or B7.2 on the APC [465].

Given that the kinetics of DC activation are rapid (chapter 3)[117, 118], questions of physiology and therapeutic potential perhaps need to be separated. While a 6h stimulation may be ideal for *in vitro* modelling of DC responses [382], a shorter period might be better for *in vivo* application, allowing a greater window of opportunity for interaction with specific T cells before the DCs reach exhaustion. It would be interesting to compare the *in vivo* responses to DCs given LPS and IL-10 for perhaps only 30 minutes before injection, to those generated by DCs first stimulated for 6 or even 24h. Would the suppression of subsequent T cell responses be stronger if the DCs were delivered earlier?

The protocol for *in vivo* use of these DCs could be optimised on several levels. The concentration of antigen used to coat the DCs, the number of cells injected and the route of administration have all been reported to affect the type of immune response

generated [466]. First, though, I wanted to confirm that IL-10 treated DCs could be effective against pathological immune responses. A mouse model of autoimmunity was used to examine their therapeutic potential.

Chapter 6 - The impact of IL-10 DCs on EAE

6.1 Introduction

Most current therapies against unwanted or pathological immune responses involve general immune suppression [467, 468]. The manipulation of dendritic cells offers a more targeted approach, potentially reducing side effects and increasing therapeutic success. Several techniques have been used to enhance the ability of DCs to elicit T cell tolerance. Dhodapkar *et al.* harvested human monocyte-derived DCs early to promote their immaturity, and with them demonstrated antigen specific inhibition of CD8+ T cell effector function [325]. Min *et al.* transfected murine DCs with Fas ligand and showed that responding T cells died by apoptosis. This suppressed allogeneic responses *in vitro* and increased allograft acceptance *in vivo* [469]. The immunosuppressive functions of IL-10 [470, 471] have also made it an attractive candidate for therapeutic manipulation. Adenoviral delivery of recombinant IL-10 into inflamed joints was able to inhibit delayed-type hypersensitivity systemically in mice [472] and retroviral transfection of DCs in culture enabled selection of a highly purified and suppressive DC population [473, 474]. Data presented in chapter 5 suggested that DCs activated by LPS in the presence of IL-10 can limit T cell responses *in vivo*. This chapter examines their therapeutic potential by asking whether they can also suppress clinical disease.

Multiple sclerosis (MS) is caused by a T cell mediated, autoimmune attack which destroys the myelin insulation of nerve axons [475]. Saltatory conduction is lost and patients suffer paralysis and sensual impairment [476]. Scotland has one of the highest frequencies of the disease worldwide: prevalence in the Lothian and Borders region was estimated in 1996 at approximately 1 in 500 [477, 478]. In mice, MS can be mimicked in certain susceptible strains either by active immunisation with myelin antigens in adjuvant [479, 480] or by passive transfer of myelin-specific T cells [481]. In both cases, the resulting disease is known as experimental autoimmune encephalomyelitis (EAE). It is an established and well characterised model of autoimmunity [482] in which the timing and course of disease are known and the

assessment of symptoms is simple and non-invasive. It is an ideal system to manipulate.

The T cell response in EAE is Th1 dominated: IFNγ and TNFα enhance T cell pathogenicity [483, 484], IL-12 contributes to disease [485] and B cells can be protective [486]. Some reports have suggested that a Th2 response inhibits disease [298, 300], but this remains controversial. Th2 cells are known to antagonise Th1 development: IL-4 prevents the differentiation of naïve T cells into Th1 effectors and IL-4, IL-13 and IL-10 can suppress IFNγ-induced macrophage activity [487, 488]. Expression of Th2 cytokines in the CNS correlates with EAE remission [489, 490]. IL-4 deficient mice are not any more susceptible to EAE than their wild-type littermates [491, 492], however, and in immunodeficient hosts, myelin specific Th2 cells induce disease [493]. After comparing EAE in IL-4 and IL-10 knockout and transgenic mice, Bettelli *et al.* suggested that it was the IL-10 component of a Th2 response that protects against disease, rather than a Th2 phenotype *per se* [492]. This is supported by a growing appreciation of the role of IL-10 dependent regulatory cells in controlling autoimmunity [494, 495] [323]. Even the importance of IL-10 is debated, however. IL-10 deficient mice suffer accelerated disease and fail to recover [492, 496]. In rats, s.c. injection of recombinant IL-10 suppressed disease [497]. In mice, adenoviral expression of IL-10 in the CNS blocked EAE induction, but systemic delivery was ineffective [498]. Repetitive injections not only failed to protect but markedly worsened disease [499].

While the involvement of IL-10 in the pathogenesis of EAE is complex, targeting its action to a specific time or place may still have considerable therapeutic power [498, 500]. In this chapter, IL-10 treated DCs are shown to be effective suppressors of EAE when injected either before or after induction of disease. The data are yet preliminary, but the effect is promising.

6.2 Approach

These experiments used a model of EAE in which disease is actively induced in C57Bl/6 mice by immunisation with the immunodominant epitope of myelin oligodendrocyte glycoprotein (MOG_{35-55} peptide). It was first described by Mendel *et al.* in 1995 [480] and is routinely used in Edinburgh by Steve Anderton and colleagues [327]. Disease was triggered by s.c. immunisation with MOG_{35-55} emulsified in CFA on day 0 of the experiment. Pertussis toxin was injected i.p., both on day 0 and again on day 2. The pertussis toxin functions as an additional adjuvant [501] and acts directly on the blood-brain barrier to allow leukocyte entry to the CNS [502, 503]. Mice showed a monophasic disease with onset of symptoms around day 9 and recovery for most mice by day 28. Mice were checked daily and scored according to the severity of their paralysis:

grade 0 = healthy
grade 1 = limp tail
grade 2 = impaired righting reflex
grade 3 = partial hind limb paralysis
grade 4 = total hind limb paralysis
grade 5 = total hind limb and partial fore limb paralysis
grade 6 = moribund or dead

To investigate the influence of IL-10 treated DCs on clinical disease, DCs were grown from C57Bl/6 bone marrow progenitors and harvested at day 7 of culture. Cells were washed, stimulated with LPS and IL-10 for 6h, pulsed with MOG_{35-55} for 90min and injected i.v. into C57Bl/6 hosts. This was done three times, either 7, 5 and 3 days before the same mice were immunised to induce EAE, as suggested by Menges *et al.* [111], or on days 0, 2 and 4 after immunisation. Mice were then monitored for the duration of disease before being sacrificed. Their spleens and inguinal lymph nodes, the nodes draining the s.c. site of immunisation, were harvested and cultured with MOG_{35-55} to assess their *in vitro* recall responses. The experimental outline is illustrated in fig 6.1.

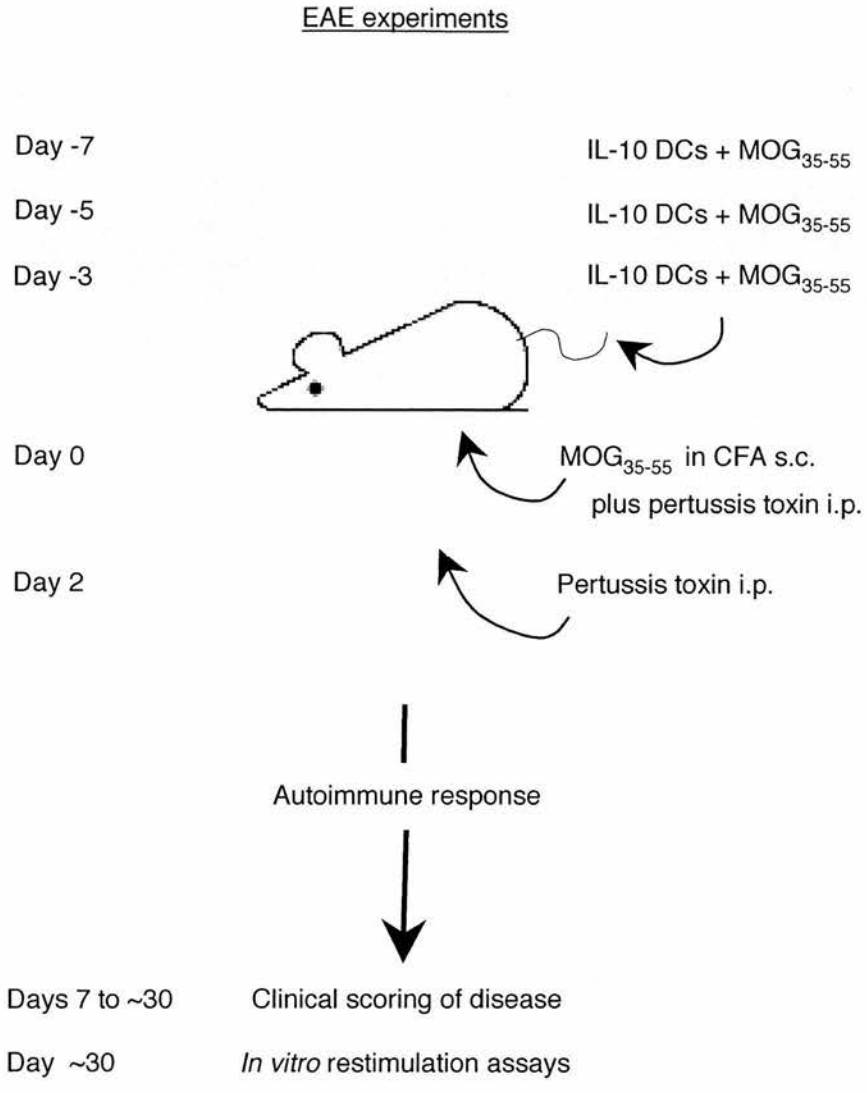

Figure 6.1 Experimental outline. A model of EAE was used to investigate whether IL-10 treated DCs could inhibit autoimmune disease. DCs were injected three times, normally 7, 5 and 3 days before EAE was induced by immunisation with MOG_{35-55} peptide and adjuvant. Disease became apparent around day 9. Mice were checked daily from day 7 and graded according to the severity of their paralysis.

6.3 Results

6.3.1 IL-10 treated DCs limit the severity of EAE

To determine whether the ability of IL-10 treated DCs to suppress T cell proliferation *in vivo* (see fig 5.14) extended to an inhibition of pathogenic T cell activity, DCs were stimulated with LPS and IL-10 before being pulsed with MOG_{35-55} and injected into mice that were later immunised to induce EAE. DCs were given 7, 5 and 3 days before immunisation. Their effect on the development of disease is illustrated in fig 6.2. Mice that were mock-injected with PBS showed a typical disease course, with clinical symptoms becoming apparent 8-9 days after immunisation, reaching maximum severity around day 14 and then steadily healing. Mice that received IL-10 treated DCs pulsed with MOG_{35-55} appeared to be protected from disease. The severity of their symptoms was reduced below that of the PBS controls, and their recovery was accelerated. Unexpectedly, a similar suppression of disease was also achieved with IL-10 treated DCs that had not been deliberately coated with peptide (fig 6.2).

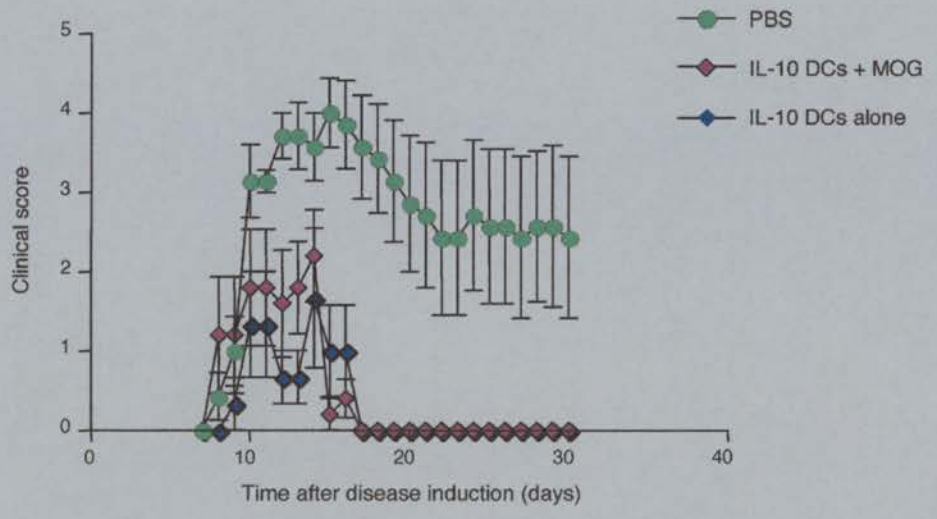

Figure 6.2 Pretreatment with IL-10 DCs suppresses EAE disease. DCs were harvested at day 7 of culture, stimulated in the presence of IL-10 and pulsed (IL-10 DCs + MOG) or not (IL-10 DCS alone) with MOG_{35-55}. The DCs were injected into mice 7, 5 and 3 days before they were immunised to induce EAE. The severity of disease was recorded daily using a discrete clinical scoring system where grade 0 is healthy and grade 6 is moribund (see Materials and Methods, chapter 2). The data is shown as the mean score within each group, ± SEM. For the PBS controls, n=7; for IL-10 DCs + MOG, n=5; for IL-10 DCs alone, n=3. The disease burdens for both DC groups were significantly less severe than that of the PBS controls (P=0.0001 for both groups, using the Mann Whitney rank sum test). This experiment was performed twice; the repeat is illustrated in figure 6.6.

6.3.2 In vitro restimulation responses

Recovery from EAE has been associated with expression of the cytokines IL-4 and IL-10 [492, 504]. To test whether the suppression of disease seen in fig 6.2 correlated with a Th2 bias, lymph node and spleen cells were harvested from these mice and their proliferation and cytokine production measured in response to restimulation *in vitro*. In keeping with the milder paralysis that they experienced, cells from mice that had received IL-10 treated DCs either with or without antigen proliferated less when rechallenged with MOG_{35-55} than those from control mice (fig 6.3). This was clear both in lymph node and spleen but again, there was little difference between the two DC groups.

The lack of proliferation seen in restimulation cultures from mice given IL-10 DCs could reflect either a smaller *in vivo* T cell expansion, leaving fewer specific T cells in each well of the *in vitro* assay, or an initially equivalent expansion that, perhaps through anergy or suppression, displays reduced proliferation on restimulation. MHC:peptide-tetramer technology (reviewed in [505]) may soon be able to distinguish the two possibilities directly, but for now, some direction can perhaps be taken from the cytokine profiles of the cultures (fig 6.4). Cells from control mice secreted IFNγ but little IL-4, consistent with a Th1 mediated disease [483, 484], although interestingly the splenocytes also produced some IL-10. Cells from mice that received IL-10 DCs secreted less IFNγ but without any concomitant increase in IL-4 production and, if anything, a loss of IL-10. The explanation for their reduced severity of disease does not appear to be a Th2 skew. The general lack of cytokine production is similar to that seen when DO11.10 T cells originally activated by IL-10 treated DCs were restimulated *in vitro* (see fig 4.6) and, as discussed in chapter 4, it is also a characteristic of T cell anergy [289, 296].

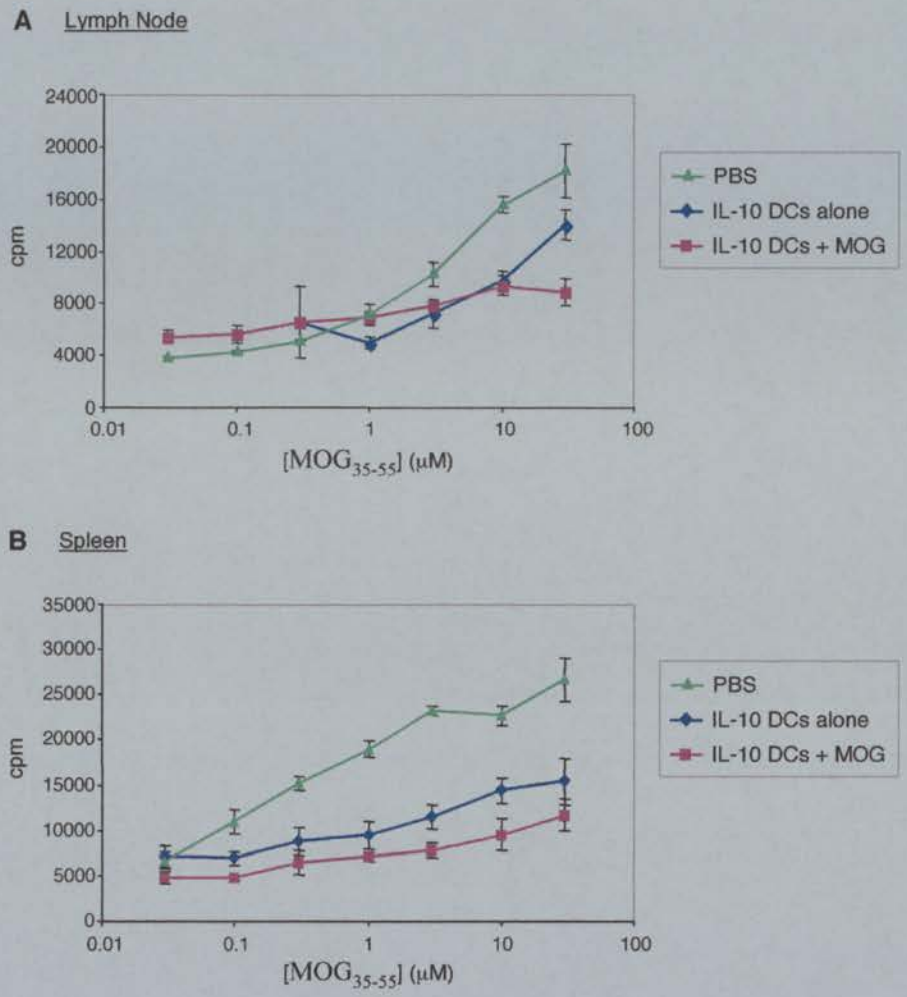

Figure 6.3 Restimulation in vitro. DCs were stimulated in the presence of IL-10 and pulsed, or not, with MOG_{35-55}. They were injected into mice 7, 5 and 3 days before immunisation to induce EAE. The disease course for each group is shown in figure 6.2. After recovery, mice were killed on day 30 and their spleens (A) and inguinal LNs (B) collected. Cells were pooled within groups, plated with graded doses of MOG_{35-55} and proliferation measured by 3H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM.

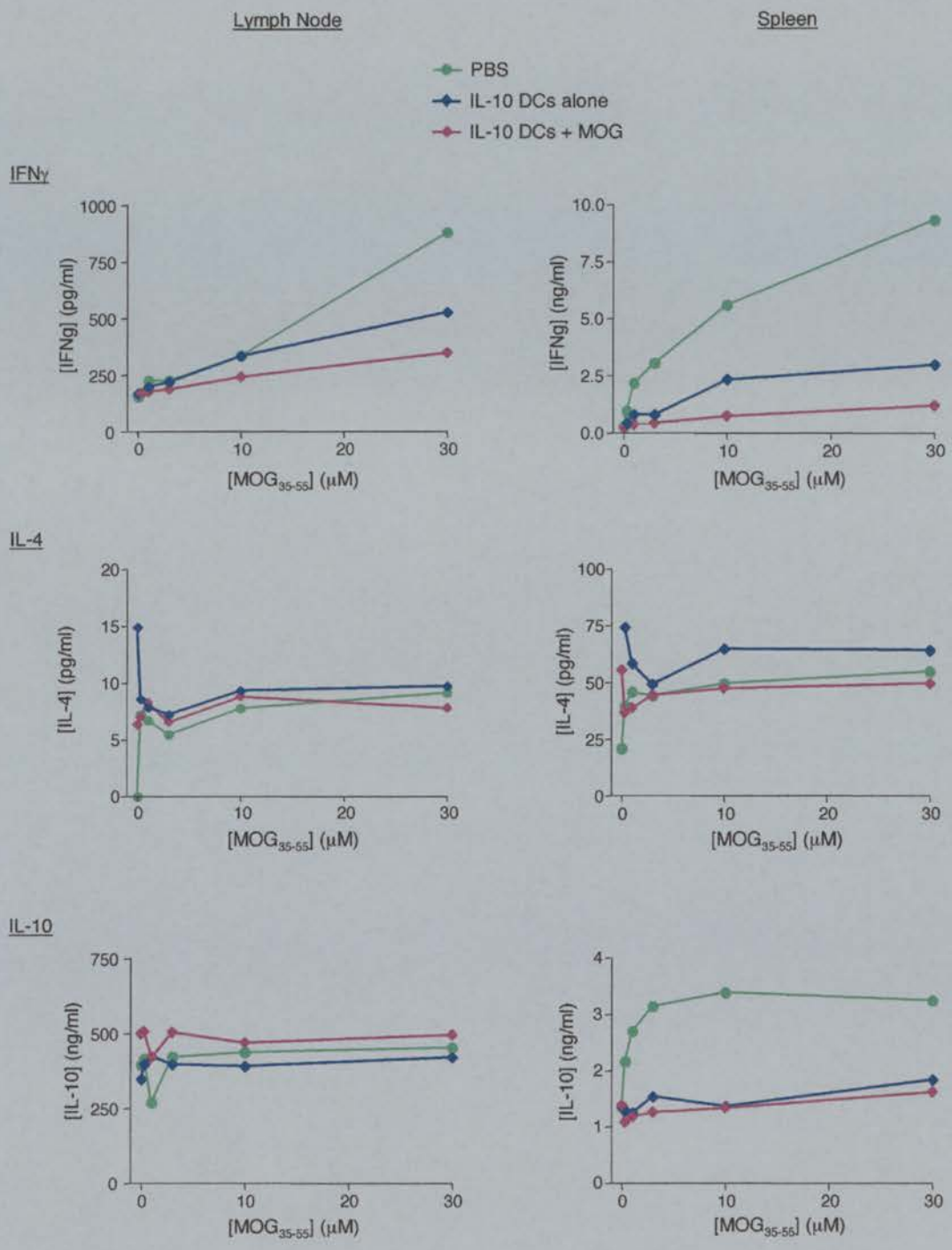

Figure 6.4 Cytokine profiles. DCs were stimulated in the presence of IL-10 and pulsed, or not, with MOG_{35-55}. They were injected into mice 7, 5 and 3 days before EAE induction. The disease course for each group is shown in figure 6.2. After recovery, mice were killed on day 30 and their spleens and inguinal LNs collected. Cells were pooled within groups, plated with graded doses of MOG_{35-55} and their cytokine production measured by a cell based Elisa method. Data is shown as the mean of duplicate wells.

6.3.3 IL-10 treated DCs are effective after disease induction

While the suggestion that pre-treatment with IL-10 DCs can reduce the severity of EAE is encouraging, particularly in its support of the suppression of DO11.10 T cell responses described in chapter 5, its therapeutic relevance is limited by the difficulty in identifying people at risk of multiple sclerosis before symptoms occur. To move towards an assessment of the potential of IL-10 DCs to treat rather than prevent EAE, the DC injections were delayed and given instead on days 0, 2 and 4 of the experiment, alongside the CFA and pertussis immunisations that provoke disease. Their impact on the severity of symptoms is shown in fig 6.5. Disease was much milder in mice that received IL-10 treated DCs coated with MOG_{35-55} than in the PBS controls: onset was delayed, peak severity was reduced and recovery was accelerated. IL-10 DCs without antigen also delayed the onset of disease slightly, but the total disease burden was not significantly reduced below that of the controls (fig 6.5). DCs activated in the presence of IL-10 can reduce the severity of EAE even when delivered after the induction of disease, and in this case it appears to be an antigen specific effect.

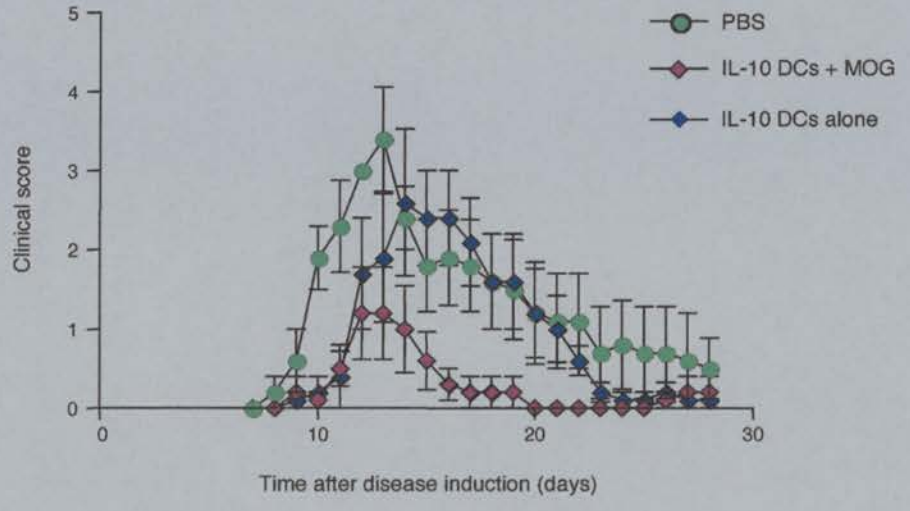

Figure 6.5 IL-10 treated DCs given after disease induction. DCs were harvested at day 7 of culture, stimulated in the presence of IL-10 and pulsed (IL-10 DCs + MOG) or not (IL-10 DCS alone) with MOG_{35-55}. The DCs were then injected into mice on the same day as immunisation to induce EAE and at 2 and 4 days afterwards. The severity of disease was recorded daily and the data is shown as the mean score of the 5 mice in each group ± SEM. IL-10 DCs + MOG significantly reduced the disease burden below that of the PBS controls ($P<0.0001$, using the Mann-Whitney rank sum test), but IL-10 DCs alone did not ($P=0.1$). This experiment was performed just once.

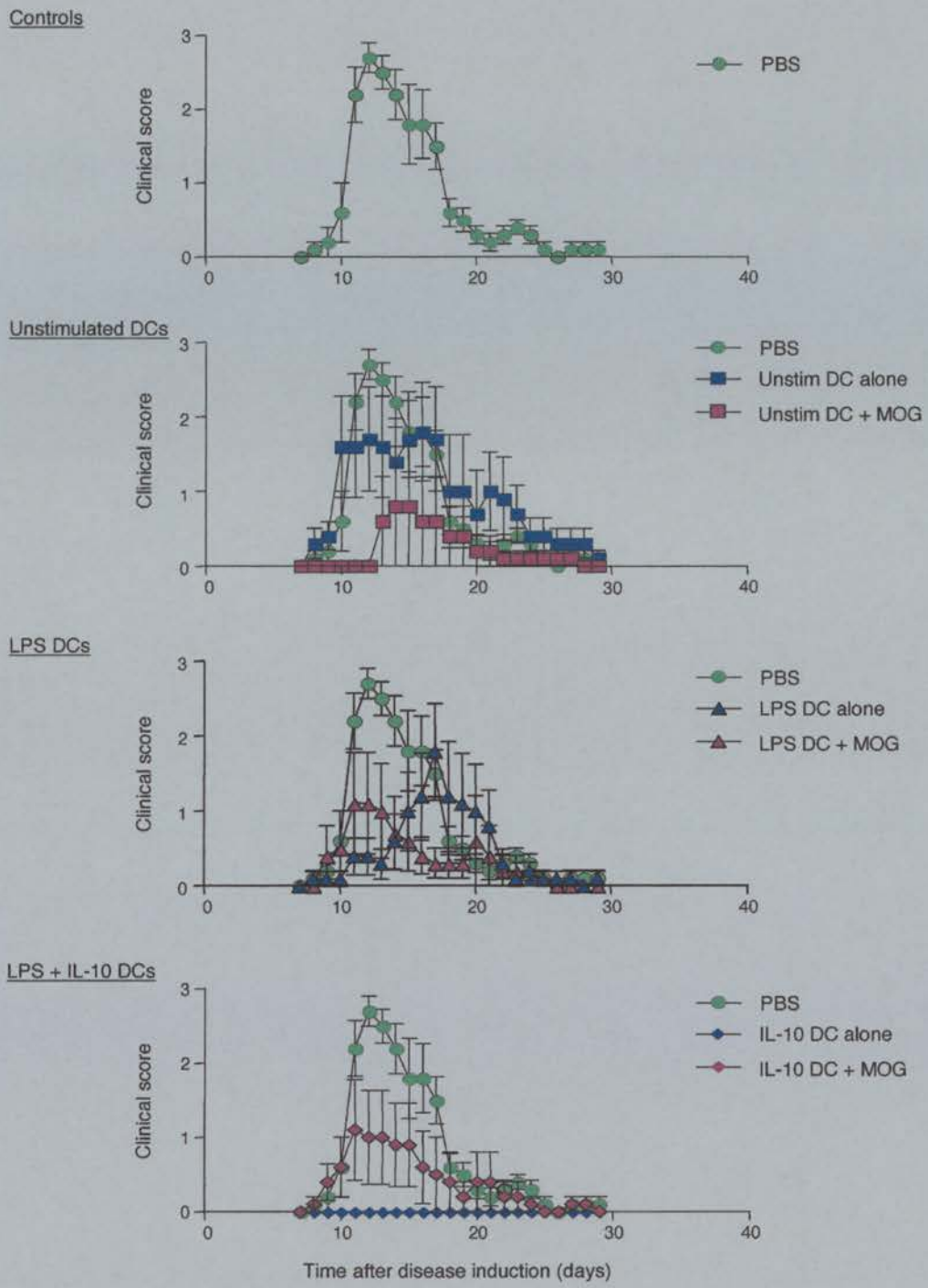

Figure 6.6 Disease suppression is non-specific. DCs were harvested at day 7 of culture and replated in medium alone, with LPS or with LPS and IL-10 together. Cells were then pulsed,or not, with MOG_{35-55} and injected into mice 7, 5 and 3 days before they were immunised to induce EAE. The severity of disease was recorded daily, and the data is shown as the mean score of 5 replicate mice ± SEM. The only groups to experience disease burdens significantly below that of the PBS controls here were unstimulated DCs + MOG (P=0.015, using the Mann-Whitney rank sum test) and IL-10 DCs alone (P<0.0001). This experiment was performed once, although some groups repeat work shown in fig 6.2.

6.3.4 Suppression is not specific

The two initial experiments using EAE to assess the impact of IL-10 treated DCs on pathogenic T cell responses suggested considerable promise (see figs 6.2 and 6.5), but a clearer understanding was needed of the specificity of their effect. The first experiment was therefore repeated with additional control groups (fig 6.6). DCs were activated with LPS and IL-10, with LPS alone or left unstimulated. Each group was pulsed with MOG_{35-55} or left without antigen, before being injected into mice 7, 5 and 3 days before immunisation with CFA. As in fig 6.2, mice that received IL-10 treated DCs experienced much milder disease than those mock-injected with PBS, and this was true irrespective of whether the DCs had been coated with MOG_{35-55} before injection. With the exception of unstimulated DCs without MOG_{35-55}, all DC groups appeared to suppress disease; the effect was neither antigen specific nor dependent on the state of DC activation. The variation within groups in this experiment was unusually high, however, as illustrated by the considerable error bars. In comparison with the PBS controls, only unstimulated DCs with MOG_{35-55} and IL-10 DCs alone achieved a statistically significant reduction in disease (fig 6.6).

6.3.5 Clinical summary

To further examine the effect of IL-10 treated DCs in EAE, data were compiled from both experiments in which mice were given DC before disease induction (table 6.7). The incidence of disease was shown to be significantly reduced by pre-treatment with IL-10 DCs, although this was more pronounced if the DCs were not pulsed with MOG_{35-55}. Those mice that received IL-10 DCs and still suffered paralysis appeared to endure a similar disease course to the PBS controls: neither the onset of symptoms nor their peak severity was significantly affected by the administration of DCs. Is EAE an all or nothing event in which IL-10 DCs encourage the regulatory balance to fall in favour of health rather than disease? Analysis of more mice will enable a stronger conclusion.

Table 6.7 EAE in mice pre-treated with IL-10 DCs

	Incidence	Cumulative disease burden per mouse	Day of onset[a]	Maximum score[b]
PBS	12/12 (100%)	44.3	9.6 ± 0.3	3.8 ± 0.4
IL-10 DCs + MOG	6/10 (60%)*	10.6***	9.3 ± 0.8+	2.7 ± 0.2×
IL-10 DCs alone	2/8 (25%)**	3.0***	9.5 ± 1.4	2.5 ± 1.4

Data are compiled from two experiments, shown individually in figs 6.2 and 6.6

[a, b] Data refer only to those mice showing clinical disease

*, ** Statistically different to that of the PBS controls. $P = 0.029$ and 0.0007, respectively, using Fisher's exact test.

*** Statistically different to that of the PBS controls. $P < 0.0001$ using the Mann-Whitney rank sum test.

+ No statistical difference to that of the PBS controls. $P = 0.45$ using the Mann-Whitney rank sum test.

× No statistical difference to that of the PBS controls. $P = 0.08$ using the Mann-Whitney rank sum test.

6.4 Discussion

The data presented in chapter 5 suggested that an *in vivo* interaction with DCs stimulated in the presence of IL-10 elicits a muted proliferation and a lasting suppression of T cell activity. This chapter demonstrates that IL-10 treated DCs can inhibit pathogenic T cells as well as DO11.10 transgenic responses; tolerance can be achieved not just in terms of limited proliferation in *in vitro* restimulation assays but also as a reduction in clinical symptoms of disease.

The ability of IL-10 treated DCs to inhibit EAE even after CFA immunisation implies that their effect is not just to anergise a cohort of responding cells, but to establish an active suppression of the T cell response. This strengthens the data

obtained in the DO11.10 bone marrow chimaeras (see fig 5.15). In support, the activated state of the DCs, at least at the time of injection, argues against induction of classical anergy due to lack of costimulation [391]. Albert *et al.* [375] and Menges *et al.* [111] have both proposed that some degree of DC maturation is needed to establish tolerance. Indeed, the protective effect of TGFβ in an EAE model in rats was attributed to stimulation of DCs [506] and blood borne DCs from patients with MS have been shown to express unusually low levels of the activation marker B7.2 [507, 508]. Immature DCs have also been associated with pathology in autoimmune diabetes [509, 510], lupus [511] and Graves' disease [512].

An argument based on the activation state of the DCs is perhaps undermined by the equivalence of the different DC populations revealed in fig 6.6, however. The experiment needs to be repeated before firm conclusions can be drawn, and it is also true that the similar effects of the three DC populations do not necessarily reflect similar mechanisms. While the unstimulated DCs used in these experiments do show some degree of maturation (see fig 3.4, for example), probably encouraged by the handling that they receive before injection [104], a comparable protocol was used by Dhodapkar *et al.* to demonstrate CD8+ T cell tolerance *in vivo* [325]. Inhibition of disease by LPS activated DCs was more surprising. A similar result has been described by Bettelli *et al.* in their comparison of EAE in IL-$4^{-/-}$ and IL-$10^{-/-}$ mice [492]. While the absence of endogenous IL-10 caused very severe EAE in response to MOG_{35-55} in CFA, the same immunisation in combination with pertussis toxin gave much milder symptoms. The additional stimulus of the second adjuvant ameliorated disease. This negative feedback could be explained by the induction of iNOS expression in strongly activated DCs, leading to nitric oxide release and consequent T cell apoptosis [513, 514].

The other issue of specificity raised in this chapter is the intriguing ability of DCs without antigen to mimic the effect of those pulsed with MOG_{35-55} (figs 6.2 and 6.6). Initially I assumed that this was symptomatic of the same high background responses that were afflicting the DO11.10 experiments at the same time (see fig 5.9). As illustrated in fig 5.10, even unpulsed DCs were generating very strong, non-specific proliferative responses and it was possible that these could overwhelm any MOG-

specific proliferation and inadvertently protect their hosts from EAE [515]. Arguing against this, the proliferation seen in *in vitro* restimulation assays from these EAE experiments was peptide dose dependent and of the two EAE experiments completed at this time (figs 6.2 and 6.5), only one appeared non-specific. Ironically, the experiment set up after the serum issue had been solved with the intention of demonstrating specificity (fig 6.6) showed particularly impressive disease suppression by unpulsed, IL-10 treated DCs.

There are more interesting explanations. Tolerogenic DCs may be able to condition their environment, perhaps through the secretion of cytokines such as IL-10 or TGFβ [112, 330, 473, 516]. As long as the release of these cytokines is independent of T cell engagement [259], IL-10 treated DCs both with and without antigen could convert surrounding APCs into tolerogenic cells. When the CFA immunisation then delivers a depot of specific antigen for endogenous presentation, the T cell response would be limited and clinical disease suppressed.

Alternatively, DCs not pulsed with peptide *in vitro* might be able to acquire antigen *in vivo*. In experiments in which mice were given DCs before EAE induction, this would require the DCs to remain alive and capable of collecting and presenting antigen 3 days after injection, when the MOG_{35-55} was delivered in the CFA immunisation. This is possible (fig 3.8)[155]. The DCs would also need to be able to access the antigen. If endogenous APCs such as Langerhans cells carried the peptide from the s.c. site of immunisation to the draining lymph node, it could be transferred there onto DCs that originated in the injected population. Such hand-over of antigen has been reported [217, 517], although data described in chapter 5 suggested that systemic delivery of DCs was relatively inefficient in reaching the lymph nodes (see fig 5.7). The administration of DCs several weeks before immunisation with MOG_{35-55} might be revealing here, since DCs are unlikely to survive that period [155] and so acquisition of antigen from the CFA deposit would be prohibited.

The concept of *in vivo* acquisition of antigen has mixed implications. On the one hand, it appears to defeat the aim of antigen-specific manipulation of the immune

system for which DCs seemed such an attractive tool. On the other, the specific antigens responsible for multiple sclerosis are not yet known, so pulsing DCs with peptide *in vitro* is not currently an option in the treatment of human disease. MOG constitutes only 0.01-0.05% of myelin proteins [518] and while there are more abundant components, such as proteolipid protein and myelin basic protein, both of which have immunodominant epitopes mapped [519, 520], there is little evidence that these are clinically relevant [521, 522]. Indeed, it has been suggested that the relapsing and remitting disease so characteristic of MS is a result of renewed waves of autoimmune attack as the T cell response switches focus from one epitope to another [523]. Although the EAE model used here is a monophasic disease, one possible explanation for the apparently greater suppression of disease by IL-10 treated DCs without MOG_{35-55} than by the same DCs coated with peptide *in vitro* (see figs 6.6 and 6.7) is that the unpulsed DCs are able to acquire an array of biologically relevant antigens from the damaged CNS *in vivo*.

This is an idea that Xiao and colleagues have tried to exploit therapeutically. Remarkably, they reported that bone marrow precursors taken from a rat that had recovered from EAE and cultured for 7 days to generate DCs, can protect other rats from subsequent attempts to induce disease [524]. The authors argue that bone marrow DCs are pulsed with CNS antigens *in vivo* and that the culture period merely serves to expand the population, although how the antigens are also multiplied is unclear. The same group have also described the use of CD8- splenic DCs, purified from rats suffering EAE and treated *in vitro* with either IFNγ or TGFβ, to inhibit the development of disease in naïve animals (quoted as unpublished data in [525]). It would be very interesting to test whether DCs isolated from the lymph nodes or spleens of the EAE mice used here, and ultimately from the blood of MS patients, could be activated in the presence of IL-10 *in vitro* and re-infused to achieve a similar suppression of disease *in vivo*.

Current therapies for MS focus on general immune suppression, although this may be mediated via the patient's endogenous DCs. Methylprednisolone, used to treat acute relapses [526], is a potent corticosteroid shown to inhibit the differentiation of monocytes into DCs [527]. Related steroids have been reported to act on immature

DCs to prevent their maturation [218, 528]. In contrast, IFNβ is administered long-term to prevent the occurrence of relapses and delay progression to disability [529]. IFNβ appears to enhance DC activation while skewing their phenotype towards one that favours the development of a Th2 response. DCs stimulated in the presence of IFNβ make little IL-12 [530] but instead release IL-10 [531] and consequently encourage a T cell reaction dominated by IL-13, IL-5 and IL-10 [532].

Activating DCs *in vitro* in the presence of IL-10 has the advantage of concentrating the action of the cytokine onto appropriate cells and with relevant timing. Systemic administration of IL-10 has been reported to inhibit collagen induced arthritis [533], but to aggravate EAE [499]. Cua *et al.* have shown that targeting the IL-10 to the site of APC:T cell interaction, by expressing recombinant IL-10 under an MHCII promoter, completely protects the transgenic mice from EAE [500]. Interestingly, this IL-10 is apparently sufficient to prevent disease: transgenic mice on an IL-$10^{-/-}$ background are also protected. T cell release of IL-10 is not required. While little IL-10 production was detected in the DCs used here (fig 3.11), IL-10 can act on DCs in an autocrine manner [212]. The transgenic IL-10 could be influencing the APCs as well as the effector T cells. Cua *et al.* go on to demonstrate that therapeutic IL-10 must be able to enter the CNS [498]. They achieve this by direct intracranial injection, but circulating lymphocytes may provide an alternative means of access. If T cell IL-10 is dispensable, B cells might be responsible [327, 534].

The data in this chapter suggest that IL-10 treated DCs can suppress pathogenic T cell responses in a mouse model of EAE, and hence reduce the severity of disease. There are interesting experiments to be done, including the optimisation of the period of DC stimulation and the timing of DC administration. Even at this preliminary stage, however, the potential for therapeutic benefit looks exciting.

Chapter 7 - Discussion and Conclusions

7.1 Summary

IL-10 is one of a number of factors reported to modulate DC function by trapping the cells in an immature state. Others include apoptotic cells [83, 464], malaria-infected erythrocytes [30] and steroidal anti-inflammatories [218], so the mechanism of regulation is relevant to immunity, tolerance, infection and therapy. Data presented in this thesis have shown that DCs activated in the presence of IL-10 do respond to LPS stimulation, downregulating antigen uptake and increasing expression of MHC and B7 (chapter 3), but that this activation is transient and does not stimulate strong T cell proliferation (chapter 4). The T cell response is instead characterised by limited proliferation and reduced cytokine production upon restimulation (chapters 4 and 5). The suppressive influence of IL-10 treated DCs is effective *in vivo* (chapter 5) and can limit the severity of disease in a mouse model of autoimmunity (chapter 6). These cells may have considerable therapeutic potential.

7.2 Discussion

7.2.1 DC activation in the presence of IL-10

The observation that a combination of LPS and IL-10 can elicit an activated DC phenotype, with high surface expression of MHCII, B7.1 and B7.2 (fig 3.5), is in contrast to the common perception of IL-10 as an inhibitor of DC maturation [216]. The discrepancy may be a result of the kinetics of DC activation: at 24h post stimulation, IL-10 treated DCs do display lower levels of MHC and B7 than equivalent cells given LPS alone (fig 3.7). The idea that DC mediated regulation of the immune response can occur despite or even because of MHC and B7 expression is not without support. A number of recent reports have described the suppression of T cell responses by phenotypically activated DCs [111, 112, 330, 375]. DC

maturation is not a single event, and the different levels of DC activation that contribute may each have a distinct influence on the consequent T cell response [113]. Indeed, the 'steady-state' migration of peripheral DCs into lymph nodes that is proposed to maintain peripheral tolerance [220], must be accompanied by some degree of DC activation in order to trigger the upregulation of CCR7 required for entry into secondary lymphoid tissue. Steinman spoke of discrete stages of DC maturation in which the change in chemokine receptors preceeded the surface stabilisation of MHCII, which in turn preceeded B7 expression (Steinman, 2002, NIH/NIAID meeting, Airlie, Virginia, USA). The separation of the DC life cycle into just two phenotypes, tolerogenic immature DCs and immunogenic mature cells, may be over simplistic [535].

7.2.2 T cell anergy

The attraction of a decisive DC activation state in response to stimulation in the presence of IL-10 is that it implies a greater functional stability than that of immature cells. Hawiger *et al.* demonstrated that the T cell tolerance induced by immature DCs could be broken by concomitant DC stimulation [328]. Nelson *et al.* have described cyclic DC activation in response to repetitive stimulation with TNFα [536], but this is hard to reconcile with the limited life-span of DCs *in vivo* [155]. It would be interesting to assess the dominance of the putative regulatory phenotype of IL-10 treated DCs. Could IL-10 treated DCs maintain tolerance to the antigen they present in the face of a strong immune response to a different peptide delivered on co-injected LPS-activated DCs?

In vitro, IL-10 treated DCs alone elicit a limited T cell proliferation (fig 4.2) that appears to leave the T cells hyporesponsive to rechallenge. Their proliferative response to restimulation is weak (fig 4.5) and they make little cytokine (fig 4.6). This phenotype is suggestive of T cell anergy [289], and analysis of T cell expression of IL-2 would have been informative. *In vivo*, particularly in the lymph node, the ability of IL-10 treated DCs to inhibit T cell proliferation in response to rechallenge was clear (fig 5.14). The stronger result *in vivo* may reflect sub-optimal culture

conditions *in vitro*. Exogenous IL-2 was added to the T cell cultures, which can overcome T cell anergy [295]. Alternatively, the explanation may lie in the different timing of the two experiments. *In vitro*, the DCs were mixed with T cells at 6h after stimulation, at the peak of MHC and B7 expression on IL-10 treated DCs. *In vivo*, the DCs were injected at this stage. Even if DC migration from the periphery to secondary lymphoid tissue is rapid, possibly within 4-6h [382], this delay may mean that the *ex vivo* IL-10 treated DCs have downregulated their expression of MHC and particularly B7 by the time they encounter specific T cells (fig 3.7). The T cell suppression observed in figs 5.13 and 5.14 may then be an *in vivo* demonstration of the same anergic tolerance seen in response to IL-10 treated DCs 24h after stimulation, originally interpreted as a result of IL-10 mediated 'inhibition' of DC maturation [205]. Powerful microscopy techniques are now becoming available which might allow direct visualisation of the levels of B7 expressed on injected DCs during T cell engagement [377, 537].

7.2.3 Regulatory T cells?

A pressing question that arises from the data presented in chapter 4 and particularly in chapter 5 is whether the hyporesponsive T cells induced by IL-10 treated DCs are also suppressive. The precedent for DC induction of regulatory T cells has been set: both Akbari *et al.* McGuirk *et al.* have recently described the ability of mature DCs to elicit Tr1 like T cells [112, 330]. In contrast to these reports, however, the IL-10 treated DCs used here did not express any detectable IL-10 (fig 3.11). While IL-10 appears fundamental for the development of Tr1 cells, other regulatory T cell phenotypes exist and other molecules are important in their differentiation (reviewed in [312, 314]). A comparison of the levels of TGFβ secreted by DCs activated with LPS alone and with LPS and IL-10 would be interesting [324].

The greatest need, however, is for some functional assessment of the responding T cell populations. The simplest approach may be to mix the two DC populations and measure the net T cell proliferation. If T cells responding to IL-10 treated DCs are anergic but not suppressive, the proliferation in the combined culture should be

equivalent to that induced by LPS-activated DCs alone. More elegantly, the recovery of T cells from DC co-cultures for subsequent titration into naïve T cell populations, either *in vitro* or *in vivo*, would enable a direct demonstration of T cell suppression. Bone marrow chimaeras are particularly attractive hosts for this type of experiment (figs 5.15 and 5.16), since the sustained thymic output of antigen specific T cells provides the naïve targets for suppression and therefore negates the requirement for successive T cell transfers. The preliminary experiment illustrated in figure 5.16 may indicate that IL-10 treated DCs can elicit an active T cell suppression. Although encouraging, the 5 day interval between primary immunisation and secondary challenge may have been too short to allow the accumulation of a significant naïve T cell population [538, 539]. Increasing the period from 5 days to several weeks may be revealing.

7.2.4 Physiological context

The data in this thesis suggest that LPS and IL-10 together drive a tolerogenic DC phenotype that can render T cells hyporesponsive to rechallenge. The combination of LPS and IL-10 is important: DCs given IL-10 alone do not respond to subsequent stimulation [540] and fully mature DCs are no longer affected by IL-10 addition [117]. It is perhaps an unusual partnership, requiring either the presence of IL-10 in the periphery or of LPS in the secondary lymphoid tissues. Both situations do occur. In the liver, hepatocyte secretion of IL-10 may act on resident DCs to dampen local immune responses [201]. In the skin, keratinocyte expression of IL-10 is markedly enhanced by contact sensitisation or UV irradiation [85, 86]. The intracellular stores that these cells accumulate mean that damaged keratinocytes may spill IL-10 into the surrounding microenvironment. An infected cut therefore provides a good opportunity for simultaneous LPS and IL-10 signals, but a tolerogenic response to this situation is unlikely to be desirable. IL-10 production by keratinocytes may instead be related to the suppression of immune reactivity against harmless environmental antigens or commensal bacteria that live on the skin [39].

The presence of LPS in secondary lymphoid tissue normally indicates a systemic infection, either due to direct inoculation into the bloodstream or to the failure of the immune system to contain the infection at the site of entry. Clinically, systemic infections are serious [541] and it is perhaps counter-intuitive to discuss tolerance induction at the same time as an urgent need for antibiotics. The life-threatening pathology of septic shock syndrome or meningococcal septicaemia is associated with extremely high levels of circulating IFNγ and TNFα [542]. The ability of splenic or lymph node DCs to respond to concomitant LPS and IL-10 by promoting T cell tolerance or suppression may be critical to regulating the balance of effective immunity and minimal immune damage. Indeed, splenic B cells have been shown to react to IL-12 by releasing IL-10, which suggests that activated B cells in secondary lymphoid tissues can downregulate Th1 responses by dampening the stimulatory capacity of DCs [271]. Any such B cell regulation would have to act on DCs resident in the spleen or lymph nodes, or on those arriving from the periphery as part of a sustained influx. Data shown in fig 3.10 suggests that, while DCs given LPS for 24h are resistant to the effect of IL-10, at 6h post-stimulation they remain responsive. The window of opportunity during which the DC must receive signals from both LPS and IL-10 in order to assume a tolerogenic phenotype may be sufficient to include the time needed to travel from the periphery to the draining lymph node.

7.2.5 Therapeutic promise

Whatever its physiological setting, the capacity of LPS and IL-10 to elicit a DC phenotype that limits T cells responses has considerable therapeutic promise. The clinical applications of dendritic cells are becoming widely appreciated (reviewed in [543]), although the majority of patient trials to date have focussed on the ability of DCs to enhance anti-tumour immunity [345] [544]. In mice, DCs can elicit complete tumour rejection [433]; in man, similar efficacy can be achieved but as yet without the same consistency [545]. The use of cytokine conditioning to manipulate DC function offers the potential to direct a range of different immune responses. DCs cultured in GM-CSF and TGFβ have been shown to prolong survival of experimental

heart allografts [546] and those grown in GM-CSF and IL-4 reduce the incidence of type 1 diabetes in non-obese diabetic (NOD) mice [547]. Results presented here have demonstrated that DCs activated in the presence of IL-10 can generate a limited T cell response that leaves the T cells hyporesponsive to subsequent challenge (chapter 4). These DCs are effective *in vivo* (chapter 5) and can reduce the severity of disease in a mouse model of multiple sclerosis (chapter 6). The data are preliminary but promising. The early clinical trials of DCs as cancer therapy have served to confirm the safety and feasibility of using protocols based on antigen pulsing of cultured DCs to combat disease [545, 548].

7.2.6 Therapeutic challenges

One of the major hurdles in translating data from animal models into human relevance is the need not just to prevent disease, but to treat established symptoms. Fig 6.4 demonstrates that IL-10 treated DCs are effective in suppressing EAE when given at the time of disease induction, but it would be valuable to delay DC administration until the onset of symptoms and even into established disease. Both mice that lack B cells and those whose B cells are IL-10 deficient suffer a severe, non-remitting form of EAE [327], and such animals might provide a means of assessing the ability of IL-10 treated DCs to promote recovery. A comparison of the efficacy of DCs when used as prophylaxis, to treat active disease and to prevent relapses would be interesting, both to assess potential clinical benefit and to understand more about the influence of the different immune system environments into which the DCs are delivered.

In multiple sclerosis, successive relapses have been associated with epitope spreading and renewed waves of autoimmune attack [523]. A critical question then is whether administration of IL-10 treated DCs loaded with one antigen can suppress T cell responses to another. EAE can be induced in different strains of mice using encephalitogenic peptides derived from myelin basic protein (MBP) and proteolipid protein (PLP) as well as the MOG_{35-55} used here. Attempts to modulate disease induced with one peptide by injection of DCs pulsed with another would be

instructive. Tumour vaccination strategies have often used DCs pulsed with whole tumour lysates in order to include a range of clinically relevant epitopes [545, 548]. Oral administration of bovine myelin to multiple sclerosis patients has been reported to induce regulatory Th3 cells against epitopes of both MBP and PLP [323], but safety concerns over BSE must now prohibit this approach. The ability of DCs to pick up antigen *in vivo* is intriguing and a rigorous investigation is needed. This approach also has inherent dangers, however. Human patients will inevitably carry unrelated antigens, such as those of latent viruses or sub-clinical bacterial infections, which a scavenging DC may also be able to access and to which tolerance may be disastrous. The use of IL-10 treated DCs in deliberately infected mice would be interesting.

7.3 Conclusions

The data presented in this thesis have demonstrated that IL-10 treated DCs do respond to stimulation. They show an early upregulation of surface MHC and B7 but, even when phenotypically active, they fail to elicit strong T cell proliferation. Both *in vitro* and *in vivo*, the interaction with DCs activated in the presence of IL-10 leaves specific T cells hyporesponsive to rechallenge. In a model of autoimmunity, these DCs reduce symptoms of disease. Together, the data suggest that, rather than inhibiting DC maturation, IL-10 directs an active DC phenotype that can regulate immune responses. *In vitro* manipulation of DC function can influence the outcome of immune responses *in vivo*.

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